Novel Mechanisms of Antihelminth Immunity

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Declaration

I, Lewis James Entwistle, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Intestinal helminths are highly prevalent worldwide, infecting approximately a third of the world's population, causing significant host morbidity. With no current vaccines, a limited number of effective chemotherapeutic drugs available and the emergence of drug-resistant helminths, it is essential to further our understanding of the mechanisms of antihelminth immunity. Our current understanding of antihelminth immunity places the type 2 immune response at the forefront of protection, with type 2 cytokines orchestrating and activating a plethora of immune and non-immune cells to mediate parasite expulsion. The naturally occurring intestinal helminth *Heligmosomoides polygyrus* establishes a chronic infection in many inbred naïve mice, with resistance to a challenge infection established following drug-cure. This experimental model allows us to identify novel mechanisms of drug-induced resistance, relative to susceptibility.

In this thesis, we utilised next generation sequencing technology to identify two novel mechanisms of antihelminth immunity. Firstly, we determined that the enzyme phospholipase A_2 group 1B (PLA_2g1B) is an endogenous anthelmintic, upregulated in intestinal epithelial cells of resistant mice. We demonstrated that PLA_2g1B was essential for resistance to *H. polygyrus* and that PLA_2g1B directly cleaves phospholipids off infective *H. polygyrus* larvae. Secondly, we identified that the microRNAs miR-99a-5p, miR-148a-3p and miR-155-5p were upregulated in mice resistant to *H. polygyrus* during infection and were also essential for functional immunity. In summary, we have identified and characterised two novel mechanisms of antihelminth immunity and propose a model of tissue memory, essential for acquired resistance to *H. polygyrus*.

Impact Statement

The analysis, discovery and insight presented in this thesis further our understanding of antihelminth immunity. Utilising RNA sequencing, we identified and describe two novel mechanisms of immunity to intestinal helminth infection. These findings have the potential to benefit other basic, translational and clinical researchers in the fields of parasitology and immunology. Furthermore, these findings may also impact the pharmaceutical industry and improve human health by providing new therapeutic opportunities in the rational design of anthelmintic drugs and/or the manipulation of microRNA (miRNA) expression.

Specifically, the vast RNA sequencing datasets created and analysed in this thesis provide an abundance of high quality, high resolution data from mice susceptible and resistant to an experimental intestinal helminth infection. These datasets are publically available and knowledge of this will be disseminated upon publishing of these results in scientific journals. Further mining of these transcriptomic datasets has the potential for the discovery of further novel mechanisms of antihelminth immunity. This will therefore benefit basic and translational researchers in the field of antihelminth immunity.

The discovery that phospholipase A_2 group 1B (PLA_2g1B) acts as an endogenous anthelmintic and is critical for protective immunity to intestinal helminth infection furthers our understanding of important antihelminth responses. Furthermore, the demonstration that PLA_2g1B cleaves phospholipids from infective larvae has revealed a completely new mechanism of antihelminth immunity. This discovery should spur interest in identifying the specific roles of larval phospholipids in infection, host-parasite interaction and larval development amongst the basic parasitology and immunoparasitology research communities. Moreover, further elucidation of PLA_2g1B's effect on intestinal helminths and the role of parasite-phospholipids in helminth infection and health would be of interest in the pharmaceutical industry. Specifically, in the design of synthetic helminth-specific molecules to recapitulate the effects of endogenous PLA_2g1B, leading to the generation of new anthelmintics. The discovery of PLA_2g1B as an endogenous anthelmintic could therefore impact treatment strategies of intestinal helminth infection, thus improving human health.

As part of this thesis, we may have identified a discrepancy between pharmacological inhibition of miRNAs and genetic deletion studies of miRNAs. This discrepancy is particularly important in the translation of basic research using genetic miRNA-deficient models into pharmacological inhibition for potential use in the clinic. We also demonstrate that inhibition of a suite of miRNAs abrogated protective immunity to intestinal helminth infection, whereas individual miRNA inhibition did not. This highlights the complex nature of miRNAs *in vivo* cooperating to regulate specific biological functions and pathways. These insights into miRNA-regulated antihelminth immunity will benefit basic researchers with interests in immunoparasitology, immunology and miRNA biology.

Finally, our data provides insights into the phenomenon of 'tissue memory' being both present and essential in protection against intestinal helminth infection. Our findings should stimulate important questions in the realm of 'tissue memory' that are of interest to the wider community of basic, translational and clinical researches.

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“A scientist in his laboratory is not a mere technician: he is also a child confronting natural phenomena that impress him as though they were fairy tales” – Marie Curie

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Abbreviations

Abbreviation	
1^o / 2^o	Primary / Secondary
A. duodenale	*Ancylostoma duodenale*
aaMφ(s)	Alternatively activated macrophage(s)
AB-PAS	Alcian blue - periodic acid schiff
Ago	Argonaute
APC	Antigen presenting cell
BCR	B cell receptor
BEC	*S*-(2-boronoethyl)-1-cysteine
BMDM	Bone marrow-derived macrophage
C. elegans	*Caenorhabditis elegans*
C1 / C2 / C3	Cluster 1 / Cluster 2 / Cluster 3
cDC1s	Classical type 1 dendritic cells
cDC2s	Classical type 2 dendritic cells
CDP(s)	Common dendritic cell precursor(s)
CILP	Common innate lymphoid progenitor
CLP	Common lymphoid progenitor
cLT	Cysteinyl leukotriene
cPLA2	Cytosolic phospholipase A_2
CSF1	Colony-stimulating factor 1 / macrophage colony stimulating factor (M-CSF)
CSF1R	Colony-stimulating factor 1 receptor / macrophage colony stimulating factor receptor (M-CSFR)
CSF2	Colony-stimulating factor 2 / granulocyte-macrophage colony stimulating factor (GM-CSF)
CSF3	Colony-stimulating factor 3 / granulocyte colony stimulating factor (G-CSF)
DALYs	Disease Adjusted Life Years
DC(s)	Dendritic cell(s)
Dgcr8	Digeorge syndrome critical region gene 8
DNMT	DNA methyltransferase
EoP(s)	Eosinophil-restricted progenitor(s)
ES	Excretory-secretory
FACS	Fluorescence-activated cell sorting
GALT	Gut-associated lymphoid tissue
GMP(s)	Granulocyte-monocyte myeloid precursor(s)
H. contortus	*Haemonchus contortus*
H. polygyrus / H.p.	*Heligmosomoides polygyrus*
HDL	High-density lipoprotein
HES	*H. polygyrus* excretory-secretory antigens
HSCs	Haematopoietic stem cells
ICS	Intracellular cytokine staining
IEC(s)	Intestinal epithelial cell(s)
IKK-β	IκB kinase

IL-	Interleukin
IL-4R	IL-4 receptor
ILC(s)	Innate lymphoid cell(s)
ILC1/2/3	Group 1/2/3 Innate lymphoid cell
IPA®	Ingenuity Pathway Analysis®
iPLA2	Calcium-independent phospholipase A_2
LC-MS	Liquid chromatography–mass spectrometry
LC-MS/MS	Liquid chromatography–mass spectrometry/ mass spectrometry
LDL	Low-density lipoprotein
Lgr5	Leu-rich repeat-containing G protein-coupled receptor 5
LNA	Locked nucleic acid
LPC	Lysophosphatidylcholine
LT(s)	Leukotriene(s)
LTi	Lymphoid tissue inducer
M cell(s)	Microfold cell(s)
MCP(s)	Mast cell progenitor(s)
Mcp1/6/7	Mast cell protease 1/6/7
MDCK	Madin-Darby canine kidney
MHC	Major histocompatibility complex
miRNA	MicroRNA
MLCs	Memory lymphocyte clusters
mLN	Mesenteric lymph nodes
MMP(s)	Matrix metalloprotease(s)
$MPPs^{type2}$	Type 2 multipotent progenitors
mRNA	Messenger RNA
MS	Mass spectromery
N. americanus	*Necator americanus*
N. brasiliensis / *N.b.*	Nippostrongylus brasiliensis
NET(s)	Neutrophil extracelluler trap(s)
NK	Natural killer
nt	Nucleotides
NTDs	Neglected tropical diseases
PAF	Platelet activation factor
PAF-AH(s)	PAF acetylhydrolase(s)
PC	Phosphatidylcholine
pDCs	Plasmacytoid dendritic cells
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PG(s)	Prostaglandin(s)
PLA_2	Phospholipase A_2
PLA_2g1B	Phospholipase A_2 group 1B
PLA_2g2A	Phospholipase A_2 group 2A
PLA_2g4A	Phospholipase A_2 group 4A
PMA	phorbol 12-myristate 13-acetate
pre-miRNA	Precursor miRNA

pri-miRNA	Primary miRNA
PS	Phosphatidylserine
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative real-time polymerase chain reaction
Relmα	Resistin-like alpha
Relmβ	Resistin-like beta
RISC	RNA-induced silencing complex (RISC)
RNA	Ribonucleic acid
Rx	Drug-cured/treatment
SEM	Standard error of the mean
snoRNA	Small nucleolar RNA
$sPLA_2$	Secretory phospholipase A_2
$sPLA_2R$	Secretory phospholipase A_2 receptor
STH	Soil transmitted helminthiasis
T cell	T lymphocyte
T. circumcincta	*Teladorsagia circumcincta*
T. muris	*Trichuris muris*
TA	Transit-amplifying
TCR	T cell receptor
Th	T helper
TIMPs	Tissue inhibitors of MMPs
TLR(s)	Toll-like receptor(s)
TNFα	Tumor necrosis factor α
TRBP	TAR RNA-binding protein
Treg(s)	T regulatory cell(s)
TSLP	Thymic stromal lymphopoietin
TSLPR	TSLP receptor
UTR	Untranslatable region
VLDL	Very low-density lipoprotein
WT	Wild-type
Lgr5	Leu-rich repeat-containing G protein-coupled receptor 5

Chapter 1. Introduction

1.1 Intestinal helminths

Helminths are a class of large, multicellular organisms that are both free-living and parasitic. Intestinal helminth infections are highly prevalent worldwide, infecting approximately a third of the world's population, and represent a large part of the 17 neglected tropical diseases (NTDs) (WHO, 2012). As well as humans, intestinal helminth infections are also extremely common in livestock. As a result, intestinal helminth infections are responsible for significant health and economic burdens (Bartsch et al., 2016, Fitzpatrick, 2013, Bethony et al., 2006). There are two main phyla of helminths: the nematodes (also known as roundworms) and the platyhelminths (also known as flatworms). The nematodes comprise of the intestinal worms (often referred to as soil-transmitted helminths) and the filarial worms that cause lymphatic filariasis and onchocerciasis. The platyhelminths comprise of the flukes (or trematodes), such as the schistosomes, and tapeworms (or cestodes) (Hotez et al., 2008).

1.1.1 Soil-transmitted helminthiasis

Soil-transmitted helminthiasis (STH) is highly prevalent in tropical and subtropical areas and often associated with impoverished conditions and poor sanitation, contributing to transmission by faecal contamination. The most common human soil-transmitted helminths are *Ascariasis lumbrocoides* (roundworm), *Trichuris trichiura* (whipworm), *Necator americanus* (hookworm), *Ancylostoma duodenale* (Hookworm) and *Strongyloides stercoralis* (threadworm); with many individuals often polyparasitised (infected with more than one species of parasitic worm) (Hotez et al., 2008). Left untreated, intestinal helminths often establish chronic, multi-year infections, despite not replicating in the host (with the exception of *Strongyloides stercoralis*).

In 2015, The World Health Organisation estimated that 883 million children are in need of treatment for STH (2011). Despite rarely inducing host mortality, many host morbidities are associated with chronic STH, causing an estimated global loss of 5.3

million Disease Adjusted Life Years (DALYs) (2016). Morbidities, including anaemia, growth stunting, malnutrition, fatigue and poor cognitive development, can be more severe and have a greater impact on children, particularly in reducing school attendance and impairing educational performance (Miguel and Kremer, 2004). These morbidities are primarily due to parasitism by the intestinal worms, feeding on the host's intestinal mucosa, submucosa, blood and ingested food.

STH is currently treated with chemotherapeutic anthelmintic drugs, such as ivermectin, albendazole and praziquantel. Many countries and global programmes have adopted mass deworming strategies, outlined by the WHO, in an attempt to control human helminthiases and eliminate morbidity. However, chemotherapeutic treatment is not prophylactic, only curing current infections, therefore failing to provide long lasting immunity. In addition, drug-resistant helminths have been identified in livestock (Kaplan and Vidyashankar, 2012), highlighting the danger of mass drug administration.

With no current licenced preventative vaccines, the development of antihelminth vaccines are essential in providing long lasting, prophylactic protection against infection. Unfortunately, the pipeline of antihelminth vaccine antigens are profoundly modest. This is a reflection of the huge scientific challenge to successfully identify relevant helminth antigens, with significant problems including limitations in the mining of helminth genomes, lack of translation in current animal models and the absence of strong correlates of protection for accelerating clinical development (Hotez et al., 2016). Despite these difficulties, there are currently two hookworm antigens and three schistosome antigens in clinical trials for antihelminth vaccines (Hotez et al., 2016).

With an over reliance on chemotherapeutic drugs, the emergence of drug-resistant helminths and stalling vaccine efforts, it is essential that we further our understanding of antihelminth immunity in the hope of identifying new therapeutic targets and treatment strategies.

1.1.2 Animal models of soil-transmitted helminthiasis

Much of our understanding of intestinal helminth infection has originated from the use of laboratory animal models. In particular, mouse models of *Heligmosomoides polygyrus*, *Nippostrongylus brasiliensis, Trichuris muris* and *Trichinella spiralis* have been studied extensively. In addition, intestinal helminths of larger mammals, such as sheep and pigs, have also been studied.

Each of the mouse intestinal helminths studied in the laboratory have different routes of infections, life cycles and niches, but all at some point reside in the intestinal tract, where they mature, become sexually active and produce eggs. The subtleties between the different helminth models have enabled the identification and description of fundamental immune responses to helminth infections, as well as highlight differences between specific species. Our current understanding of the immune response to intestinal helminth infection is outlined in section **1.2**.

1.1.2.1 *Heligmosomoides polygyrus*

Heligmosomoides polygyrus is a naturally occurring intestinal helminth of the mouse, first isolated from wild mice in 1939 (Spurlock, 1943). It shares the same phylogenetic Order, Strongylida, as the human hookworm parasites *N. americanus* and *A. duodenale* as well as the ruminant parasites *Haemonchus contortus* and *Teladorsagia circumcincta* (Gouy de Bellocq et al., 2001). *Heligmosomoides polygyrus* has been referred to by a variety of names over the past 70 years, such as *Nematospiroides dubius*, *Heligmosomoides polygyrus bakeri* and *Heligmosomoides polygyrus polygyrus*, but here will be referred to as *H. polygyrus* throughout.

H. polygyrus is a strictly enteric helminth and is subsequently transmitted by the faecal-oral route (Ehrenford, 1954). *H. polygyrus* eggs, measuring approximately 77 microns long by 45 microns wide, are secreted in the host faeces at the 8-16 cell stage, becoming fully embryonated after 8-10 hours before hatching after a further 16 hours at temperatures of 23-28^{o}C (Ehrenford, 1954). The first stage (L1) larvae, undergo two developmental moults (or ecdysis) to become L2 then infective L3

larvae, measuring 480-563 microns long. The final ecdysis, from L2 to L3 larvae, is not complete outside the host as the L3 larvae retain the cuticle from the L2 larvae as a protective sheath, marking the transition from the free living to the parasitic stage (Sommerville, 1957, Ehrenford, 1954). Following oral infection with L3 *H. polygyrus* larvae, the larvae enter the digestive tract where they are exsheathed in the stomach of host, losing the cuticle retained from the L2 stage (Sommerville and Bailey, 1973). The L3 larvae then pass into the small intestine where they penetrate the intestinal mucosa and embed into the *muscularis externa* within 48 hours of infection (Camberis et al., 2003, Ehrenford, 1954). Here the larvae undergo rapid growth, resulting in two developmental moults (into the L4 then L5 stage), and re-emerge into the lumen as adult L5 worms after approximately 10 days to feed on host intestinal tissue (Camberis et al., 2003, Bansemir and Sukhdeo, 1994). In the lumen the adult worms wrap around the villi to anchor themselves, favouring the duodenum and anterior jejunum due to increased villus length (Bansemir and Sukhdeo, 1996). Adult females are significantly larger than adult male *H. polygyrus* worms, measuring 18-21 and 8-10 mm respectively (Ehrenford, 1954). In the lumen, adult worms form mating pairs and produce eggs which are released into the environment in the host's faeces, thus continuing the life cycle (Ehrenford, 1954, Camberis et al., 2003). The life cycle of *H. polygyrus* is summarised in **Figure 1.1**.

H. polygyrus is frequently used as a model to investigate host immunity to intestinal helminth infections in an attempt to delineate mechanisms of susceptibility and resistance (Allen and Maizels, 2011, Maizels et al., 2012b). Unlike the mouse-adapted rat parasite *N. brasiliensis*, *H. polygyrus* establishes a chronic infection in susceptible strains of mice (Ehrenford, 1954), enabling the modelling of human chronic STH in an evolutionary adapted host. With a strictly enteric lifecycle, *H. polygyrus* is also a suitable model for studying the development of intestinal immune response in isolation, without the complexity of immune responses in other organs (such as the lung following *N. brasiliensis* infection or the muscle following *T. spiralis* infection).

Following primary (1^o) infection of *H. polygyrus*, the length of time the infection can persist is dependent upon the strain of mouse. SJL and SWR mice are able to clear 1^o *H. polygyrus* infection most rapidly, in 4-6 weeks of infection; BALB/c, DBA/2 and NIH mice in 6-8 weeks; C57BL/6, C57BL/10 and 129/J mice in 8-20 weeks; whilst

CBA C3H, SL and A/J mice take more than 20 weeks to expel 1^{o} infection (Ben-Smith et al., 2003, Reynolds et al., 2012) as well as failing to mount a protective response to secondary (2^{o}) challenge infection (Enriquez et al., 1988). Genetic comparison of these strains identified the major histocompatibility complex (MHC) H-2 loci as a significant genetic factor which mediates differences in susceptibility (Behnke and Robinson, 1985). Specifically, studies with H-2 congenic C57BL/10 mouse strains elegantly demonstrate that mice with H-2^{s}, H-2^{d} or H-2^{q} haplotypes expelled *H. polygyrus* 1^{o} infection more rapidly, whereas mice with H-2^{b} or H-2^{k} haplotypes, backcrossed onto the fast expelling BALB/c background, were unable to expel as quickly (Behnke and Wahid, 1991). Importantly, establishment of 1^{o} infection after two weeks was equal between all strains (Behnke and Wahid, 1991), suggesting that protective immune response and expulsion mechanisms were generated at different rates depending on haplotype. A study comparing the fast expelling SWR and slow expelling CBA strains identified significant candidate genes associated with resistance using quantitative trait loci mapping, including MHC, *Il9* (encoding Interleukin (IL)-9), *Mcp6*, *Mcp7* (encoding mast cell protease 6 and 7, respectively) and several trefoil factors (Behnke et al., 2003). Gender also influences susceptibility to *H. polygyrus* infection, with female mice of all strains clearing 1^{o} and, to a lesser extent, 2^{o} infections quicker than male mice (Van Zandt et al., 1973, Dobson and Owen, 1978, Wahid and Behnke, 1993).

If 1^{o} *H. polygyrus* infection is cleared using anthelmintic drugs, such as Pyrantel Embonate, the majority of mouse strains are resistant to 2^{o} challenge infection due, in part, to the generation of an effective memory immune response (Finkelman et al., 1997). Interestingly, during 2^{o} infection the majority of parasites are trapped and killed in the intestinal wall during development (acquired resistance) (Chaicumpa et al., 1977), unlike in 1^{o} infection where more resistant strains clear the infection once the adult worms have emerged into the intestinal lumen (natural resistance) (Behnke and Wahid, 1991). The mechanisms of killing and expulsion in both acquired and natural resistance may be subtly different as the parasite is located in two distinct areas of the intestine.

A third model of resistance to *H. polygyrus* has also been developed, utilising vaccination. Mice are able to elicit sterile immunity to 1^{o} infection following immunisation with *H. polygyrus* excretory-secretory antigens (HES) alongside an

alum adjuvant, trapping and killing the parasites in the intestinal wall (Hewitson et al., 2011, Hewitson et al., 2015).

Intestinal helminths have immunomodulatory properties, enabling establishment of chronic infection by defusing host immune responses and without inducing host mortality. *H. polygyrus* has been used as a mouse model to study the immunomodulatory properties of intestinal helminths as it has evolved to establish chronic infection in the mouse. A TGFβ-like protein is secreted by *H. polygyrus* which induces the differentiation of regulatory T cells (Tregs) which dampen the immune response to *H. polygyrus*, allowing a chronic infection to establish (Maizels et al., 2012a, Grainger et al., 2010, Rausch et al., 2009). Furthermore, adult *H. polygyrus* secrete exosomes containing parasite-derived miRNAs which are transferred to the host to supress type 2 immunity (Buck et al., 2014) (see section **1.3.3.4**).

1.1.2.2 *Nippostrongylus brasiliensis*

N. brasiliensis is a naturally occurring intestinal helminth of rats, which has been adapted for use in mice, and is used in the laboratory as an acute model of human hookworm infection.

Unlike *H. polygyrus*, *N. brasiliensis* penetrates the skin and migrates through the lung before traveling up the trachea, down the oesophagus and finally reside in the small intestine. Eggs are secreted in the host faeces into the environment at the 16-21-cell stage (Camberis et al., 2003). In the environment, the eggs hatch after 24 hours and undergo two developmental moults from L1 to L2 larvae after a further 24 hours and L2 to infective L3 larvae within a further 24 hours (Camberis et al., 2003). The infective L3 larvae undergo full ecdysis in the environment and therefore do not retain a protective sheath, unlike *H. polygyrus (Camberis et al., 2003)*. The infective L3 larvae penetrate the skin within five minutes of contact, although the majority of experimental infections are given by subcutaneous injection, and burrow into the epidermis before migrating into the bloodstream (Haley, 1962, Camberis et al., 2003). Within 11 hours of infection the L3 larvae can be found in the lung after migrating through the pulmonary blood vessels. Here, in the lung, the larvae undergo another developmental moult into L4 larvae where they remain until approximately

4-5 days post infection (Haley, 1962, Camberis et al., 2003). At this point, the L4 larvae migrate up the airways of the lung and trachea, down the oesophagus and through the stomach before establishing in the duodenum and jejunum. In the small intestine, the L4 larvae undergo their final developmental moult into L5 adult worms which feed on host tissue, mate and produce eggs (Camberis et al., 2003) (Haley, 1962). Eggs are found in the host faeces from 6 days-post infection, peaking between days 8 and 9 before declining as the worms are expelled by day 11 (Camberis et al., 2003).

For experimental use, mice are normally infected with 200-500 L3 larvae by subcutaneous injection. Bolus infections greater than 1000 L3 larvae in a 1^{o} infection causes significant host morbidity and occasional mortality (Camberis et al., 2003). However, mice can cope with infections greater than 1000 L3 larvae upon 2^{o} infection (Camberis et al., 2003), as most worms are killed.

1.1.2.3 *Trichuris muris*

T. muris is a naturally occurring intestinal helminth of the mouse and is used in the laboratory to model *Trichuris trichiura* infections in humans.

T. muris is also a strictly enteric helminth and has no free living larval stages, unlike *H. polygyrus* and *N. brasiliensis*. Infection occurs upon host ingestion of infective eggs. *T. muris* eggs accumulate in the cecum and after ninety minutes post infection the L1 larvae hatch from the eggs. The interaction of the host microbiota is critical for the induction of egg hatching, with bacterial type 1 fimbrae being essential (Hayes et al., 2010). Upon hatching, L1 larvae penetrate into the wall of the proximal colon, including the cecum, where it resides in the epithelial layer and undergoes three more developmental moults to the L4 stage (Klementowicz et al., 2012). By 32 days-post infection, adult worms are present, at which point eggs are secreted and can be found in the host faeces (Klementowicz et al., 2012). During the course of development, the parasite gets larger and the posterior of the whipworm extends into the lumen of the gut. The anterior part of the whipworm remains buried in parasite-modified epithelial cells, in contact with the epithelial cell cytoplasm from where they absorb nutrients (Tilney et al., 2005). Eggs secreted in the host faeces require

approximately 2 months to embryonate and become infective (Klementowicz et al., 2012).

Mice with different genetic backgrounds vary in susceptibility to *T. muris* infection. Similar to genetic susceptibility to *H. polygyrus*, the H-2 allele of the MHC influences susceptibility to *T. muris*; with H-2^k and H-2^d haplotypes being more susceptible than the H-2^q and H-2^b haplotypes, which expel the parasites faster (Else et al., 1990, Else and Wakelin, 1988).

Infective dose size can also influence the balance between susceptibility and resistance to *T. muris* infection through altering the polarisation of the immune response. Specifically, low dose infections of less than 40 eggs develop a chronic infection due to the development of a susceptible-associated type 1 immune response (Bancroft et al., 1994). Whereas a high dose infection of more than 200 eggs are expelled through the initiation of a type 2 immune response (Bancroft et al., 1994). Moreover, a high dose infection also renders mice resistant to subsequent high or low dose infections through the generation of a memory Th2 response (Bancroft et al., 2001).

1.2 Immunity to intestinal helminth infection

Our current understanding of the immune response to intestinal helminths, underpinning susceptibility and resistance, has developed predominantly from observations made in laboratory animal models as well as clinical observations. These observations have placed type 2 immunity at the forefront of protection against intestinal helminth infection. In this section, the immune response to intestinal helminths is outlined, drawing on information gathered using mouse models of *H. polygyrus*, *N. brasiliensis* and *T. muris* infection (**Figure 1.2**).

Upon entry into the intestine, helminth presence and damage to local tissue is detected by intestinal epithelial cells, which secrete a variety of alarmin-type cytokines, including IL-25, IL-33 and TSLP, which activate immune cells and initiate immunity (Zaph et al., 2007, Gerbe et al., 2016, von Moltke et al., 2016, Howitt et al., 2016). These alarmin cytokines support the activation and expansion of a suite of innate and adaptive immune cells (Fallon et al., 2006, Saenz et al., 2008, Saenz et

al., 2010). Both group 2 innate lymphoid cells (ILC2s) and antigen sampling dendritic cells (DCs) process and present helminth antigens and promote T helper (Th)2 cell differentiation. $CD4^+$ Th2 cells are essential in orchestrating an effector type 2 immune response (Grencis et al., 1991, Hashimoto et al., 2009, Urban et al., 1991a) in the small intestine through the secretion of the type 2 cytokines IL-4, IL-5 and IL-13 (Oeser et al., 2015). These cytokines act on a plethora of immune and stromal cells, inducing immunological and physiological changes. IL-4 and IL-13 driven alternatively activated macrophages contribute significantly to various aspects, including helminth trapping, immune cell activation and wound healing (Anthony et al., 2006, Esser-von Bieren et al., 2015, Herbert et al., 2004, Gratchev et al., 2001). Similarly, the activation of mast cells (Hepworth et al., 2012) and basophils (Herbst et al., 2012, Schwartz et al., 2014) and the mobilisation of granulocytes, such as eosinophils (Patel et al., 2009, Morimoto et al., 2004) and neutrophils (Sutherland et al., 2014, Chen et al., 2014, Morimoto et al., 2004), contribute to parasite trapping and support cytokine-mediated effector pathways. The development of memory Th2 cells, as well as IL-4, IL-5 and IL-13 induced B cell expansion, class switching and the production of parasite specific antibodies (McCoy et al., 2008), are essential for long-lived immunological memory and immunity to subsequent infection. IL-4 and IL-13 driven physiological changes culminate in a 'weep and sweep' response in an attempt to physically remove the parasite from the intestinal lumen, involving increased epithelial turnover (Cliffe et al., 2005), goblet cell hyperplasia with mucus hypersecretion (Hasnain et al., 2011, Hasnain et al., 2010) and smooth muscle contraction (Vallance et al., 1997). Although the exact mechanism of helminth killing is unclear, epithelial cell-derived Relmβ was shown to have direct antihelminth effects, inhibiting the feeding of adult worms in the intestine (Herbert et al., 2009).

1.2.1 Adaptive immunity

1.2.1.1 T cells

T lymphocytes (T cells) develop from common lymphoid progenitor (CLP) cells in the bone marrow, before exiting through the lymphatic system into the thymus. Here, the CLP cells undergo multiple stages of development, leading to the formation of

functional α and β T cell receptors (TCRs) (mediated by RAG genes), commitment to CD4 or CD8 fate and establish central tolerance (reviewed in (Shah and Zuniga-Pflucker, 2014)) producing a constant supply of peripheral T cells. Mature, naïve $CD4^+$ T cells exit the thymus and migrate through lymphatic vessels to tissue-draining lymph nodes, such as the intestine-draining mesenteric lymph nodes (mLN). Naïve T cell differentiation into specialised T helper (Th) subsets requires presentation of an antigen on MHC class II molecules from an antigen presenting cell (APC) to the αβTCR, co-receptor stimulation (T cell CD28 interacting with CD80/86 receptors on the APC) and a cytokine signal, usually from a primed innate immune cell, to direct the differentiation to a specific Th subtype. Th2 cell differentiation requires exogenous IL-4, enabling the secretion of the canonical Th2 cytokines IL-4, IL-5 and IL-13 (Le Gros et al., 1990).

$CD4^+$ cells are essential for immunity to both *H. polygyrus* (Urban et al., 1991a), *N. brasiliensis* (Urban et al., 1995) and *T. muris* (Koyama et al., 1995, Else and Grencis, 1996), with $CD4^+$ Th2 cell differentiation observed upon intestinal helminth infection (Svetic et al., 1993, Rausch et al., 2008, Bancroft et al., 1994). Both gene expression and protein secretion of Th2-associated cytokines were seen to be increased in the mLN following *H. polygyrus* infection (Finney et al., 2007, Svetic et al., 1993), *T. muris* infection (Faulkner et al., 1998, Grencis et al., 1991, Bancroft et al., 1994) and *N. brasiliensis* infection (Ishikawa et al., 1998). The production of the type 2 cytokines IL-4 and IL-13 are essential in orchestrating the protective immune response to intestinal helminth infection.

Resistance to 2^o *H. polygyrus* challenge infection is reduced upon administration of an IL-4 blocking antibody and completely abolished upon blocking the IL-4 receptor (IL-4R), suggesting a partial or compensatory role for IL-13 mediated-protection (Urban et al., 1991b). Administration of IL-4 in complex with anti-IL-4 antibody, to extend the half-life of IL-4, is sufficient to induce expulsion of 1^o *H. polygyrus* infection in BALB/c and SCID mice, which lack T and B cells (Urban et al., 1995). This data indicates that the protective effect of exogenous IL-4 complex treatment was not to potentiate Th2 cells, but rather to increase the IL-4Rα-dependent innate effector responses and activate IL-4Rα-driven pathways in stromal cells, outlined in section **1.2**.

Resistance to *T. muris* infection was also abolished upon genetic deletion of IL-4 in C57BL/6 mice (Bancroft et al., 1998, Bancroft et al., 2000) and upon genetic deletion of IL-13 in 129 mice (Bancroft et al., 1998). However, when IL-4 was deleted in the more resistant BALB/c mouse, resistance to *T. muris* was maintained in an IL-13 dependent manner (Bancroft et al., 2000).

Interestingly, despite IL-4 complex rescuing immunity to *N.* brasiliensis infection following CD4 depletion (Urban et al., 1995), IL-4 is dispensable for resistance to *N. brasiliensis* mouse infection (Urban et al., 1998). Instead, IL-13 production in *Il4*-deficient mice and signalling through IL-4Rα and Stat6 confer resistance (Urban et al., 1998, McKenzie et al., 1998).

Despite IL-4 being essential for Th2 differentiation (Le Gros et al., 1990), as a type 2 effector cytokine, IL-13 has been described to be equally or more important in mediating type 2 effector responses through IL-4Rα (Zhu et al., 1999, Li et al., 1999, Finkelman et al., 1999). The observations seen in *T. muris* and *N. brasiliensis* mouse infection models suggest that, in particular intestinal helminth infections, IL-13 is sufficient for resistance to infection.

The Th2 cytokine IL-5, which supports eosinophilia, is not required for protective immunity to intestinal helminth infections. Blocking of IL-5 with an antibody did not compromise immunity to *H. polygyrus* or *N. brasiliensis* (Urban et al., 1991b) and genetic deletion of IL-5 did not abrogate resistance to *T. muris* infection (Dixon et al., 2006).

H. polygyrus infection also induces hybrid Th1/2 cells, expressing both Gata-3 and T-bet and capable of producing IL-4, IL-13 and IFNγ (Peine et al., 2013). These Th1/2 hybrid cells are stably maintained as memory cells *in vivo* and resist reprogramming into classic Th1 or Th2 cells. Functionally, Th1/2 hybrid cells can support both inflammatory type 1 and type 2 immune responses but cause significantly reduced immunopathology than Th1 or Th2 cells, respectively (Peine et al., 2013). Whether Th1/2 hybrid cells are important in resistance to intestinal helminth infection is currently unclear. However, these cells are likely to be beneficial to intestinal helminths as they prevent excessive host pathology and therefore may contribute in the establishment of chronic infection.

As well as inducing effector T cells, Tregs are also induced upon intestinal helminth infection. This phenomenon has been predominantly studied in the laboratory using *H. polygyrus*, with infection potently inducing Tregs (Wilson et al., 2005, Finney et al., 2007, Rausch et al., 2008, Pelly et al., 2017). The induction of Tregs during intestinal helminth infection is thought to limit the host's Th2 response, otherwise directed at the parasite, and preventing intestinal pathology, collectively establishing a chronic infection (Rausch et al., 2009). However, Treg depletion did not induce expulsion of *H. polygyrus* during a 1^{o} infection, despite an increase in Th2 cells and their associated cytokines, IL-4 and IL-13 (Rausch et al., 2009).

1.2.1.2 B cells

B lymphocytes (B cells), like T cells, also develop from common lymphoid progenitor (CLP) cells in the bone marrow. They exit the bone marrow into the circulatory system as immature B cells and migrate to the spleen to continue their development. In the spleen, immature B cells differentiate in to naïve, follicular or marginal zone B cells. The maturation of B cells requires the rearrangement of their antigen-specific receptor, the B cell receptor (BCR), which is also mediated by RAG genes. Marginal zone B cells develop into short lived plasma cells upon contact with antigen, whereas follicular B cells develop into memory B cells or plasma cells in the germinal centre (reviewed in (Pieper et al., 2013)).

B cells are responsible for producing antibodies that facilitate the clearance of a variety of pathogens and antigens. They also regulate immune responses through antibody-independent mechanisms. Upon activation through Toll-like receptor (TLR) stimulus, B cells express costimulatory molecules and can act as APCs, up-taking specific antigens via their BCR before processing and presenting the antigen to CD4^{+} T cells via MHC class II (Linton et al., 2003, Linton et al., 2000). B cells are therefore capable of shaping the primary and memory T cell response through antigen presentation (Lund et al., 2006). B cells can also influence immunity through the secretion of the cytokines, such as TNFα, IL-6 and IL-10 (Shen and Fillatreau, 2015).

Elevated IL-4 levels during intestinal helminth infections promote B cell class switching and production of IgE and IgG1 antibodies (Katona et al., 1991, Urban et

al., 1991a, McCoy et al., 2008). In B cell-deficient mice, resistance is abrogated to 1^{o} *T.* muris infection in normally resistant C57BL/6 mice and to 2^{o} challenge infection of both *H. polygyrus* and *T. muris* (Blackwell and Else, 2001, Liu et al., 2010, Wojciechowski et al., 2009). The mechanism by which B cells are protective in 2^{o} *H. polygyrus* infection is currently unclear: two studies demonstrate that in B cell-deficient mice, protective immunity is not defective due to impairment of a Th2 response (Liu et al., 2010, McCoy et al., 2008); however, the findings of another study suggested that B cell-deficient mice had an impaired Th2 response following *H. polygyrus* infection, with the *H. polygyrus*-specific memory Th2 response reliant upon B cells-derived IL-2 and TNFα (Wojciechowski et al., 2009). Similar conclusions were drawn in regard to 1^{o} *T. muris* infection in B cell-deficient mice, with B cell deficiency compromising the Th2 response (Blackwell and Else, 2001). Interestingly, B cells are dispensable in mediating protection from 1^{o} or 2^{o} infection with *N. brasiliensis* mouse infection, with B cell-deficient mice still mounting a strong Th2 response (Liu et al., 2010).

The role of antibodies in mediating protection against intestinal helminth infection remains incompletely understood. Clinical vaccine efforts are currently stalling as identifying relevant antigens is proving to be a difficult challenge (Hotez et al., 2016). The role of antibodies in mediating protection against laboratory intestinal helminth infection differs between animal models. Despite the induction of IgE in WT mice, protective immunity was still intact in B cell-deficient mice following *N. brasiliensis* infection (Liu et al., 2010), suggesting that antibodies do not play a critical role in expulsion of *N. brasiliensis*.

T. muris infection also induces antibody production, with IgM, IgG, IgA and *T. muris*-specific IgG1 antibodies seen from 14 days-post infection (Koyama et al., 1999, Blackwell and Else, 2002). Despite abrogated resistance to *T. muris* infection in B cell-deficient mice being restored by adoptive transfer of IgG from previously infected resistant donor mice (Blackwell and Else, 2001), adoptive transfer of CD4^{+} T cells alone into SCID mice (lacking T and B cells) results in expulsion of *T. muris* (Else and Grencis, 1996). This suggests that antibodies can confer resistance to *T. muris* infection, but they are not essential.

Antibodies are essential in mediating protection against *H. polygyrus*, with infection inducing IgA, IgE and IgG1 isotype production (McCoy et al., 2008, Hewitson et al., 2011). *H. polygyrus*-induced IgE and IgG1 required MHC class II-dependent $CD4^+$ T cell help prior to class switching (McCoy et al., 2008). 1^o *H. polygyrus* infection also induces a nonspecific, polyclonal antibody response, whereas 2^o infection induces *H. polygyrus*-specific antibodies (McCoy et al., 2008). Nonspecific, polyclonal IgG functions to limit parasite fecundity, whereas affinity matured, parasite-specific IgG1 and IgA promote protective immunity upon passive transfer to naïve mice (McCoy et al., 2008, Wojciechowski et al., 2009). Despite the production of both *H. polygyrus*-specific and nonspecific IgE following infection, both IgE and IgM have no role in protective immunity (McCoy et al., 2008, Wojciechowski et al., 2009). Vaccination of naïve mice with HES and an alum adjuvant induces the generation of HES-specific IgG1 antibodies which promote sterile immunity to subsequent *H. polygyrus* infection (Hewitson et al., 2015, Hewitson et al., 2011). It is unclear whether similar titres of HES-specific antibodies develop naturally and contribute to resistance.

Parasite-specific IgG1 antibodies are believed to confer protection to *H. polygyrus* through the trapping of L3/L4 larvae in the intestinal wall. *H. polygyrus*-specific antibodies were shown to promote adherence of macrophages to L3 larvae *in vitro*, reducing larval motility, and promote larval trapping in the intestinal wall *in vivo* (Esser-von Bieren et al., 2013). Antibody-mediated trapping of larvae in the tissue was dependent on CD11b (involved in complement-mediated immune complex binding (Sanchez-Madrid et al., 1983)) and IgG receptor FcγRI (CD64) expression on macrophages, with CD11b mediating macrophage adherence to the larvae and FcγRI promoting macrophage IL-4-independent expression of Arginase 1 (*Arg1*) (Esser-von Bieren et al., 2015, Esser-von Bieren et al., 2013). However, FcγR-deficient mice and complement component C3-deficient mice are still able to expel 2^o challenge *H. polygyrus* infection (McCoy et al., 2008), therefore suggesting this mechanism of helminth trapping is not essential for protective immunity. Data from the vaccine-induced immunity model to *H. polygyrus* suggests that the protective effects of parasite-specific IgG1 are not mediated by activation of FcγRI, but instead rely on IL-4Rα- and IL-25-dependent effector cells (Hewitson et al., 2015).

These data suggest that the role of *H. polygyrus*-specific antibodies differs between vaccine-mediated and acquired immunity: IgG1-mediated binding of larvae by

macrophages is not essential for vaccine-induced immunity; instead IgG1 is critical for sequestering HES products which allows for effector immune cells to promote *H. polygyrus* expulsion.

1.2.2 Innate immunity

1.2.2.1 Innate lymphoid cells

Innate lymphoid cells (ILCs) differentiate from the common innate lymphoid progenitor (CILP) in the bone marrow and are present in mucosal tissues, such as the lung and gut (Geiger et al., 2014, Zook and Kee, 2016). ILCs mirror $CD4^+$ Th cell subtypes, both in transcription factor expression and their cytokine secretion profile. However, ILCs are distinguished from Th cells by their expression of the thymocyte marker Thy1.2 and their lack of common lymphocyte lineage markers, such as somatically rearranged antigen receptors (*e.g.* TCR or BCR) (Zook and Kee, 2016). Group 1 (ILC1), group 2 (ILC2) and group 3 (ILC3) innate lymphoid cells make up the ILC family, including lymphoid tissue inducer (LTi) cells and conventional natural killer (NK) cells (reviewed in detail in (Zook and Kee, 2016)).

ILC2s, first described as nuocytes and innate helper type 2 cells, populate mucosal sites as well as the tissue draining lymph nodes (Neill et al., 2010, Fallon et al., 2006, Pelly et al., 2016). Unlike other ILCs, ILC2s express Sca-1 and KLRG1 which correlate with Gata-3 expression (Zook and Kee, 2016). They also express the IL-25 receptor (*Il17rb*) and IL-33 receptor (ST2/*Il1rl1*), making them responsive to epithelial alarmin cytokines (Fallon et al., 2006, Zook and Kee, 2016). ILC2s also express MHC class II, allowing them to present antigen (Neill et al., 2010). Upon IL-25 or IL-33 stimulation, ILC2s produce the type 2 cytokines IL-5 and IL-13 (Neill et al., 2010, Walker et al., 2013), and IL-4 following leukotriene D_4 stimulation (Pelly et al., 2016), placing ILC2s in the centre of type 2 immunity.

Indeed, ILC2s contribute to the protective type 2 immune response to intestinal helminths. In response to *N. brasiliensis* infection, *Il25*-deficient mice fail to activate ILC2s, correlating with delayed worm expulsion in the intestine (Fallon et al., 2006). In T cell-deficient mice, recombinant IL-25-dependent activation of ILC2s was sufficient to mediate accelerated expulsion of *N. brasiliensis* (Fallon et al., 2006).

Activated ILC2 survival is dependent upon IL-9, with IL-9R-deficient mice displaying reduced numbers of ILC2s following *N. brasiliensis* infection which result in impaired type 2 cytokine production and delayed worm expulsion (Turner et al., 2013). In addition, trefoil factor 2 (Tff2)-deficient mice, which do not produce IL-33 protein from epithelial cells or inflammatory DCs upon *N. brasiliensis* infection, fail to efficiently expel the parasite (Wills-Karp et al., 2012). Although the authors attribute this delayed expulsion to ineffective Th2 differentiation and cytokine production from $CD4^+$ T cells to drive type 2 effector responses (Wills-Karp et al., 2012), it is also plausible that abrogation of an IL-33 response to *N. brasiliensis* infection prevented ILC2 expansion and activation and ILC2-derived type 2 cytokine release (Neill et al., 2010), although this was not addressed. Nevertheless, ILC2s are essential for efficient expulsion of *N. brasiliensis* mouse infection.

Treatment with recombinant IL-25 or IL-33 promoted expulsion of *T. muris* in mice genetically susceptible to *T. muris* infection (Owyang et al., 2006, Humphreys et al., 2008). However, the effect of IL-25 or IL-33 was dependent on T cells as susceptibility was maintained in SCID mice treated with recombinant IL-25 (Owyang et al., 2006, Humphreys et al., 2008). Whether IL-25- or IL-33-dependent activation of ILC2s was required for protective Th2 differentiation during *T. muris* infection is unclear.

In contrast to *N. brasiliensis* infection, the expansion of ILC2s in T and B cell-deficient mice was only able to provide partial immunity to *H. polygyrus* infection, despite significant production of type 2 cytokines and initiation of downstream type 2 effector responses (Pelly et al., 2016). ILC2 expansion and activation by mast cell-derived IL-33 may be responsible for this protection (Shimokawa et al., 2017). During 1^o *H. polygyrus* infection in WT C57BL/6 mice, ILC2s facilitate Th2 differentiation in the small intestine, through the secretion of both IL-4 and IL-2 (Pelly et al., 2016), which is essential for immunity to *H. polygyrus* (Urban et al., 1991a, Urban et al., 1991b).

1.2.2.2 Macrophages

Macrophages are mononuclear, phagocytic myeloid cells which function to clear apoptotic cells, cellular debris and pathogens. Macrophages are found in almost all

tissues throughout the body, but are particularly prominent at mucosal sites, such as the lung and intestine. The majority of macrophages are derived from embryonic progenitors, which are seeded in peripheral tissues of the foetus to give rise to tissue-resident macrophages that self-maintain throughout life, with each population specifically adapted to their niche (Guilliams et al., 2014). The development and survival of macrophages is dependent on their expression of colony-stimulating factor 1 receptor (CSF1R, also known as macrophage colony stimulating factor receptor (M-CSFR)) and the cognate ligands colony stimulating factor 1 (CSF1, also known as macrophage colony-stimulating factor 1 (M-CSF)) and IL-34 (Greter et al., 2012, Wang et al., 2012). Despite presence of tissue-resident macrophages, during inflammation circulating monocytes, from the bone marrow, infiltrate inflamed tissues where they differentiate into monocyte-derived macrophages to participate in pathogen killing or tissue repair (Guilliams et al., 2014). In specific tissues and under the appropriate conditions, monocytes can differentiate into tissue-resident macrophages to replenish the population after an inflammatory event. Macrophage development and origins are thoroughly reviewed in (Guilliams et al., 2014) and (Guilliams and Scott, 2017).

The alternate activation of macrophages is a hallmark of the type-2 immune response to helminth infections. The type 2 cytokines IL-4 and IL-13 alternatively activate macrophages, inducing the expression of *Arg1* (gene encoding Arginase 1)*, Chi3l3* (gene encoding Chitinase-like 3, also known as *Ym1*) and *Retnla* (Relmα, encodes Resistin-like alpha, also known as *Fizz1*) via IL-4R-STAT6 dependent signalling (Goerdt and Orfanos, 1999, Rodriguez-Sosa et al., 2002, Dasgupta et al., 2011, Raes et al., 2002). Alternatively activated macrophages (aaMϕs) are believed to have three distinct functions: regulation of the immune response, wound healing and resistance to parasite invasion. In regard to regulating the immune response, parasite elicited aaMϕs have been shown to downregulate helminth-induced Th1 cells and subsequent immunopathology (Herbert et al., 2004).

Given the relatively large size of *H. polygyrus*, significant damage occurs when the L3 larvae penetrate into the wall of the duodenum. It is thought that aaMϕs contribute to tissue repair and wound healing by clearing debris and upregulating genes associated with tissue remodelling, such as fibronectin, matrix metalloproteases (MMPs), tissue inhibitors of MMPs (TIMPs) and several collagen types (Gratchev et

al., 2001, Sandler et al., 2003, Wilson et al., 2007). *Chi3l3* expression has also been implicated in wound healing (Liu et al., 2004b). AaMϕs accumulate around *H. polygyrus* larvae in the wall of the intestine, forming cysts or granulomas around the invading larvae (Morimoto et al., 2004, Anthony et al., 2006). IL-4Rα signalling is sufficient for the expansion of tissue-resident macrophages in the small intestine upon *H. polygyrus* infection, independent of CSF1 (Jenkins et al., 2013). The presence of memory Th2 cell-induced aaMϕs is essential for immunity against *H. polygyrus* as depletion of macrophages with clodronate-loaded liposomes prevented expulsion upon 2^{o} infection (Anthony et al., 2006). Specifically, Arginase 1 derived from aaMϕs is essential in protective immunity to *H. polygyrus*: Arginase inhibition with *S*-(2-boronoethyl)-1-cysteine (BEC) abrogated the protection during 2^{o} infection (Anthony et al., 2006); however direct antiparasitic activity has been difficult to elucidate. Chitinase, chitinase-like proteins (such as *Chi3l3*) and resistin-like family members are secreted by aaMϕs and were hypothesised to have direct effects on tissue-dwelling helminths through enzymatic degradation of helminth-specific proteins. However, direct antiparasitic effects of these aaMϕ-associated proteins have not been discovered. Instead, roles have been described in mediating type 2 immune responses (Nair et al., 2009, Cai et al., 2009). An early study identified that macrophages isolated from mice infected with *H. polygyrus* were shown to adhere to and damage exsheathed L3 larvae *in vitro*, resulting in reduced infectivity *in vivo* (Chaicumpa and Jenkin, 1978). IL-33 can also directly induce alternate activation of macrophages and IL-13 production *in vitro* and *in vivo* (Yang et al., 2013). Furthermore, adoptive transfer of *in vitro* IL-33-induced aaMϕs promoted resistance to *H. polygyrus* infection, correlating with enhanced smooth muscle hyper-contractility and increased *in situ* IL-13 production (Yang et al., 2013).

The infiltration of aaMϕs and the expression of their associated markers are also observed in the small intestine upon infection with *N. brasiliensis* (Zhao et al., 2008). Interestingly, the accumulation of macrophages in the small intestine following infection was not dependent upon T or B cells, whereas the alternate activation was. Administration of recombinant IL-13 rescued alternate activation in SCID mice (Zhao et al., 2008). Clodronate-loaded liposome depletion of both resident and recruited macrophages in the small intestine abrogated immunity to *N. brasiliensis* (Zhao et al., 2008). Whether aaMϕs and their associated molecules have any direct

antihelminth activity in this model is unclear. *N. brasiliensis*-induced smooth muscle hyper-contractility and increased muscle thickness was inhibited when macrophages were depleted or when Arginase was inhibited with BEC (Zhao et al., 2008), suggesting that aaMϕs may regulate the effects of type 2 immunity on smooth muscle cell function.

Infection with *T. muris* increased the accumulation of macrophages in the large intestine, with a greater number of infiltrating macrophages seen in resistant strains (Little et al., 2005). A protective role for macrophages has yet to be conclusively proven in *T. muris* infection. However, if a protective role does exist, it is not through the expression of Arginase 1 in aaMϕ as both genetic deletion and BEC treatment had no effect on worm burden, immune response or intestinal pathology following *T. muris* infection (Bowcutt et al., 2011).

1.2.2.3 Dendritic cells

Dendritic cells (DCs) are professional antigen presenting cells critical in priming the adaptive immune system. DCs differentiate from common DC precursors (CDPs), which originate from adult haematopoietic stem cells (HSCs), distinct from that of monocytes (Guilliams et al., 2014). Mature DC populations are broadly subdivided in to three groups based on their distinct developmental pathways: classical type 1 DCs (cDC1s), which are dependent on Batf3 expression; classical type 2 DCs (cDC2s), which are dependent on IRF4 expression; and plasmacytoid DCs (pDCs), which are dependent on E2-2 expression (reviewed in (Guilliams et al., 2014)).

DCs are present at all mucosal sites and environmental interfaces, such as the intestine, where they are able to sample their environment and detect pathogens. Upon activation by various stimuli, such as alarmin cytokines or TLR ligands, DCs uptake antigen and migrate to the local draining lymph nodes where they present antigen on MHC class II molecules to naïve $CD4^+$ T cells (MacDonald and Maizels, 2008). Presentation of the antigen to the αβTCR of the naïve T cell alone is not sufficient for T cell differentiation, but requires both co-receptor stimulation (T cell CD28 interacting with CD80/86 receptors on the DC) and a cytokine signal, such as IL-4 for Th2 cell differentiation (Le Gros et al., 1990, Kapsenberg, 2003). The source

of IL-4 is likely to originate from innate immune cells, with ILC2s, mast cells and eosinophils shown to be capable of producing IL-4 (Pelly et al., 2016, Gessner et al., 2005, Voehringer et al., 2004). These data have prompted a continuing debate regarding the cellular source of IL-4 required for Th2 differentiation.

Although the evidence presented in previous sections suggests that type 2 immune responses to intestinal helminth infection can be driven in the absence of the adaptive immune system, $CD4^+$ Th2 cells are fundamentally important for immunity to some intestinal helminths, such as *H. polygyrus* and *T. muris* (Urban et al., 1991a, Urban et al., 1991b, Koyama et al., 1995, Else and Grencis, 1996), indicating that DCs, or any other APCs, are critical for their activation.

$CD11c^+$ DCs are essential for the initiation and maintenance of Th2 cells *in vivo* (Phythian-Adams et al., 2010). However, following depletion of $CD11c^+$ DCs, type 2 effector responses, such as alternative activation of macrophages, were still intact despite abrogation of Th2 differentiation and Th2 cytokine release (Smith et al., 2012, Smith et al., 2011). Unlike our understanding of viral and bacterial sensing and activation of DCs, the manner in which DCs sense helminth infection is not completely understood. Helminth products have been shown to modulate TLR4 signalling in DCs, inhibiting both IL-12p40 and p70 protein production and promoting Th2 cell differentiation (Goodridge et al., 2004). Interestingly, deletion of MyD88, which is an essential signalling adaptor protein for TLR and IL-1 family members, had heightened protective immune responses following *H. polygyrus* infection, mounting a stronger Th2 response. These data suggest that MyD88 signalling can promote susceptibility to *H. polygyrus* (Reynolds et al., 2014a). Whether heightened immunity seen in MyD88-deficient mice is attributable to MyD88-deficient DCs is unclear. It is interesting to speculate that MyD88-deficiency prevents DC activation by TLR ligands, thus preventing IL-12 induction and therefore promoting Th2 differentiation. Promoting Th2 differentiation is essential for protective immunity to *T. muris* infection, however restriction of MHC class II expression to DCs was not sufficient to generate Th2 differentiation and cytokine production, abrogating resistance to *T. muris* (Perrigoue et al., 2009). Immunity was restored in mice with MHC class II expression restricted to DCs when IFNγ was neutralised (Perrigoue et al., 2009), suggesting that DCs promote Th2 responses, only if Th1 development is blocked.

Tumour progression locus 2(Tpl-2, also known as Map3k8 and Cot) kinase activity activates the Mek-Erk Mapk signalling pathway in response to TLR, TNFR1 and IL-1R stimulation in innate immune cells (Gantke et al., 2011). Mice with Tpl-2-deficient $CD11c^+$ DCs were resistant to 1^o *H. polygyrus* infection, with Tpl-2-deficient DCs producing more of the chemokine Ccl24. Increased Ccl24 stimulated increased accumulation of innate immune cells and Th2 cells in the small intestine, promoting acceleration of type 2 effector responses which leads to killing of the invading larvae (Kannan et al., 2017).

Taken together, these data demonstrate that DCs are essential in initiating and maintaining Th2 cell differentiation in response to intestinal helminth infections.

1.2.2.4 Mast cells

Mast cells are long-lived granulocytes which, upon appropriate stimulation, undergo degranulation and release a range of inflammatory molecules. Mast cells mature from mast cell progenitors (MCPs) in peripheral tissues, which themselves are derived from the bone marrow. Following development, MCPs enter into the circulatory system and migrate to tissues in an immature state in a regulated process that is stimulated by inflammation, leading to an increase in local tissue MCPs (reviewed in (Dahlin and Hallgren, 2015)). Unlike the lung, the intestine contains many MCPs, even in germ-free mice or mice deficient in the Rag-2 gene either alone or in combination with the IL-receptor common gamma (γ) chain (Guy-Grand et al., 1984, Gurish et al., 2001), suggesting they may play essential roles in maintaining gut homeostasis and responding to infection. Indeed, mastocytosis and activation is a hallmark of intestinal helminth infection.

Mastocytosis and elevated Mcp1 correlate with expulsion of 1^o *H. polygyrus* infection (Behnke et al., 1993, Wahid and Behnke, 1993, Ben-Smith et al., 2003, Hepworth et al., 2012). Mcp1 is hypothesised to disrupt the niche of adult *H. polygyrus* worms by increasing gut permeability by disrupting epithelial tight junction proteins (McDermott et al., 2003, Snoek et al., 2012). Mast cell-deficient mice harbour the same number of adult worms 21 days-post 1^o *H. polygyrus* infection, but worms isolated from mast cell-deficient mice deposited more eggs *in vivo* and *ex vivo* (Hashimoto et al., 2010).

In support, mice with genetically induced intestinal mastocytosis were demonstrated to have a significantly lower egg burden following *H. polygyrus* infection (Morimoto and Utsumiya, 2011). Another study demonstrated that mast cell-deficient mice were defective in expulsion of 1^{o} *H. polygyrus* infection, harbouring more intestinal worms 21 days-post infection (Hepworth et al., 2012). The reason for this discrepancy is unclear.

In addition, protective immunity was abrogated in mast cell-deficient mice following 2^{o} challenge infection (Hepworth et al., 2012). The protective role of mast cells in this study was attributed to their role in the priming of the Th2 response through promoting IL-25, IL-33 and TSLP release following infection (Hepworth et al., 2012). Supporting this type 2 priming role of mast cells, a more recent study has identified that mast cells are activated to produce IL-33 by ATP released from apoptotic epithelial cells following *H.* polygyrus infection. Mast cell-derived IL-33 promoted activation of IL-13 producing ILC2s and initiation of type 2 immune effector responses (Shimokawa et al., 2017). Taken together, these data suggest a role for mast cells in protective immunity to *H. polygyrus*.

Upon infection with *N. brasiliensis*, intestinal mastocytosis correlates with the migration of the parasite to the intestine (Uber et al., 1980). However, mast cell-deficient mice are able to expel *N. brasiliensis* (Uber et al., 1980). Similarly, following *T. muris* infection, mast cell numbers increase in the cecum (Guy-Grand et al., 1984), however antibody-mediated depletion of mast cells had no effect on worm expulsion (Betts and Else, 1999). As a result, mast cells are currently thought to be dispensable for protective immunity to *N. brasiliensis* and *T. muris*.

1.2.2.5 Eosinophils

Eosinophils are a subset of granulocytes generally associated with type 2 immune responses and are a hallmark of intestinal helminth infection. Eosinophils originate from HSCs in the bone marrow, which gives rise to eosinophil-restricted progenitors (EoPs) (Orkin and Zon, 2008). Gata1 expression and IL-5 are essential for EoP development into immature eosinophils, which subsequently leave the bone marrow and enter the bloodstream (Byström et al., 2004). IL-5 signalling triggers the

development of mature eosinophils, resulting in nucleus condensation and granule maturation (Willebrand and Voehringer, 2017). Eosinophils have a short lifespan in the circulation, however this is increased when eosinophils seed in peripheral tissues, such as the small intestine with IL-5 and, to a lesser extent, colony stimulating factor 2 (CSF2, also known as granulocyte-macrophage colony stimulating factor (GM-CSF)) being survival factors (Rothenberg, 2016, Schwartz et al., 2015). Eotaxin family proteins, eotaxin-1 (Ccl11), eotaxin-2 (Ccl24) and eotaxin-3 (Ccl26) are chemokines which bind to the chemokine receptor Ccr3, which is expressed on eosinophils, drawing them to sites of inflammation (Pease, 2006). Eosinophil development and survival is reviewed in (Uhm et al., 2012) and (Willebrand and Voehringer, 2017).

Despite induction of eosinophilia upon intestinal helminth infection (Dixon et al., 2006, Rennick et al., 1990, Urban et al., 1991b) their precise roles remain elusive. Eosinophils are not critical mediators of protective immunity. In genetic mouse models of eosinophil deficiency, immunity to *N. brasiliensis* or *T.* muris was not impaired (Knott et al., 2007, Dixon et al., 2006). Furthermore, administration of anti-IL-5 antibody, which reduces eosinophil numbers, did not impair expulsion of *N. brasiliensis* or 2^{o} *H. polygyrus* infection, despite inhibiting eosinophilia (Khan et al., 1995, Urban et al., 1991b). Moreover, helminth products have been shown to directly promote eosinophil chemotaxis (Dixon et al., 2006). Interestingly, early studies demonstrated that eosinophils isolated from infected mice can adhere to various developmental stages of *H. polygyrus* larvae/worms (Penttila et al., 1983) and cause damage to exsheathed L3 larvae *in vitro*, as measured by a loss of infectivity *in vivo* (Penttila et al., 1984). Taken together, these data suggest that eosinophils may possess antihelminth activity but are not critical mediators of immunity to intestinal helminth infection.

1.2.2.6 Neutrophils

Neutrophils, a subset of granulocytes and the most abundant immune cell population, play a crucial role in host defence. Neutrophils are characterised by their ability to act as phagocytic cells, to release lytic enzymes, produce reactive oxygen

species and other products with antimicrobial properties (reviewed in (Kruger et al., 2015)). Neutrophils reside from HSCs in the bone marrow, which differentiate into granulocyte-monocyte myeloid precursors (GMPs), before developing into mature neutrophils which exit into the circulation (Coffelt et al., 2016). Colony-stimulating factor 3 (CSF3, also known as granulocyte colony stimulating factor (G-CSF)) is the master regulator of neutrophil generation (Lieschke et al., 1994, Liu et al., 1996).

Neutrophils are present in the granulomas surrounding tissue-embedded larvae following both 1^{o} and 2^{o} *H. polygyrus* infection, however they are less prevalent in 2^{o} infection (Anthony et al., 2006, Morimoto et al., 2004, Patel et al., 2009). The decrease in neutrophils upon 2^{o} *H. polygyrus* infected mice suggests that they are not a critical cell in protective immunity, or they have undergone netosis to produce neutrophil extracellular traps (NETs) (Bonne-Annee et al., 2014, Branzk et al., 2014). Early studies demonstrated that neutrophils, as well as eosinophils, isolated from infected mice can both adhere to various developmental stages of *H. polygyrus* larvae/worms (Penttila et al., 1983) and cause damage to exsheathed L3 larvae (Penttila et al., 1984). Moreover, antibody-mediated neutrophil depletion was shown to partially compromise resistance to 2^{o} *H. polygyrus* challenge infection (Penttila et al., 1985). However, antibody-mediated depletion of neutrophils during *T. muris* infection did not abrogate protective immunity (Zaph and Artis, 2015). A more recent study illustrated a role for neutrophils in infection with *N. brasiliensis*, with *Chi3l3*-induced neutrophil recruitment limiting helminth survival at the cost of local tissue damage (Sutherland et al., 2014). Although the exact mechanism is unclear, these data demonstrate a protective role for neutrophils following helminth infection. Furthermore, following *N. brasiliensis* infection, neutrophils were shown to be essential for priming and maintaining long-lived effector aaMϕs, essential for heightened resistance to 2^{o} *N. brasiliensis* infection (Chen et al., 2014). These neutrophils were shown to adopt a ‘type 2’ phenotype, upregulating expression of *Il13*, *Il33*, *Igf1*, *Retnla* and *Chi3l3* and were essential in the alternate activation of macrophages (Chen et al., 2014).

Overall, the role of neutrophils, and NETs, have not been fully elucidated in immunity to intestinal helminth infections. However, despite clear antihelminth properties, they appear not to be critical for protection against helminth infections.

1.2.3 Stromal immunity

1.2.3.1 Epithelial cells

Epithelial cells line mucosal surfaces, such as the lung and intestine, and function as a barrier between the host and the environment. In the small intestine, there are many different subtypes of intestinal epithelial cell (IEC), all of which arise from Leu-rich repeat-containing G protein-coupled receptor 5-expressing ($Lgr5^+$) intestinal stem cells. Intestinal stem cells reside at the base of the crypts and continuously generate transit-amplifying (TA) cells. TA cells occupy the remainder of the crypt and reserve stem cells from the +4 position from the crypt base and can also repopulate the $Lgr5^+$ stem cells following injury. TA cells differentiate into various functional cells to replace the IECs lost via anoikis at the tip of the villi, with complete IEC turnover occurring every 3-5 days in the small intestine. The main subtypes of IECs are enterocytes, enteroendocrine cells, tuft cells, goblet cells and paneth cells; each with specialised functions (reviewed in (Barker, 2014)).

IECs are often in direct contact with intestinal helminths and therefore function both as critical initiators of immunity and effector cells. Epithelial cells secrete a suite of alarmin cytokines, IL-25, IL-33 and TSLP to initiate the type 2 immune response (Fort et al., 2001, Soumelis et al., 2002, Schmitz et al., 2005). Partial redundancy between these three alarmin cytokines in initiating type 2 immunity has been demonstrated (Vannella et al., 2016), although distinct roles have also been elucidated in the context of intestinal helminth infection. Mice with IL-25-deficiency or inhibition of IL-33 signalling display impaired type 2 immune responses to *N. brasiliensis* infection, resulting in impaired worm expulsion (Fallon et al., 2006, Wills-Karp et al., 2012). Recent studies identified that tuft cells are able to sense intestinal helminths through the taste-chemosensory G-protein coupled receptors. Upon helminth detection, tuft cells secrete IL-25 which activates ILC2s to secrete IL-13, which feedback to promote tuft cell hyperplasia, thus providing a feed-forward mechanism to initiate type 2 immunity (Gerbe et al., 2016, von Moltke et al., 2016, Howitt et al., 2016). Tuft cells are key in initiating the type 2 immune response to intestinal helminths. Genetic deletion of tuft cells, or key signal transduction molecules in the taste-chemosensory

pathway, abrogated protective immunity to *N. brasiliensis* (Gerbe et al., 2016). Tuft cells have also been shown to undergo IL-4Rα-dependent hyperplasia following *H. polygyrus* infection (Gerbe et al., 2016, Howitt et al., 2016), however their role in protective immunity to this mouse intestinal helminth has not been determined. Both IL-25 and IL-33 are important in initiating protective immunity upon *T. muris* infection (Humphreys et al., 2008, Saenz et al., 2010). Specifically, IL-25 promotes the accumulation of type 2 multipotent progenitors ($MPPs^{type2}$), similar to ILC2 cells, in the gut-associated lymphoid tissue (GALT), which can induce Th2 differentiation and expulsion of *T. muris* (Saenz et al., 2010). Similarly, IL-33 is expressed early following *T. muris* infection and promotes the production of Th2 cytokines, preventing an inappropriate Th1 response (Humphreys et al., 2008). Additionally, exogenous administration of IL-33 to susceptible mice promoted expulsion of *T. muris* and also induced increased intestinal pathology. However, IL-33 treatment could not induce *T. muris* expulsion if administered once chronic infection had established (Humphreys et al., 2008). Interestingly, mouse strains resistant to *T. muris* infection produce more IL-33 early in infection, when compared to susceptible strains (Humphreys et al., 2008). IL-33 has also been shown to induce the Th2-inducing alarmin cytokines TSLP from IECs (Humphreys et al., 2008). Abrogation of TSLP-induced TSLP receptor (TSLPR) signalling prevented expulsion of *T. muris* infection, due to a defective Th2 response. Instead an ineffective Th1 response was induced, promoting severe intestinal inflammation (Taylor et al., 2009). Blockade of IFNγ or IL-12p40 rescued protective immunity in *T. muris* infected TSLPR-deficient mice, restoring the Th2 response (Massacand et al., 2009, Taylor et al., 2009). The induction of a protective type 2 immune response was not dependent on TSLP following infection with either *H. polygyrus* or *N. brasiliensis*, with TSLPR-deficient mice able to expel 2^{o} *H. polygyrus* challenge infection (Massacand et al., 2009). The discrepancy in requiring TSLP for induction of type 2 immunity between these three intestinal helminths was due to their excretory-secretory (ES) products. ES products from both *H. polygyrus* and *N. brasiliensis* were able to supress DC production of IL-12p40, therefore inhibit Th1 polarisation and promoting Th2 responses, whereas ES products from *T. muris* could not (Massacand et al., 2009).

As well as sensing pathogens and initiating the immune response, IECs are also key effector cells in the type 2 immune response against helminth infections. The type 2

cytokines IL-4 and IL-13 signal via IL-4Rα on IECs induce vast changes in the architecture and physiology of the intestine. Following 2° *H. polygyrus* infection IL-4Rα-dependent changes in small intestine physiology were identified; these included increased mucosal permeability, decreased ion absorption and increased prosecretory effects in response to inflammatory mediators (Shea-Donohue et al., 2001, Madden et al., 2004). IEC-mediated changes in small intestine physiology are hypothesised to promote protective immunity by disrupting the niche of *H. polygyrus*, impairing their ability to coil around villi (Bansemir and Sukhdeo, 1996) and feed on host tissue (Bansemir and Sukhdeo, 1994). Mice resistant to *T. muris* infection have an increased proliferation and turnover of IECs. This increased turnover acts like an 'epithelial escalator' where IECs move from the bottom to the top of the crypt to be shed, expelling the IEC-embedded *T. muris* worms (Cliffe et al., 2005). This increased IEC turnover is dependent upon IL-13 signalling, not IL-4, and is inhibited by the chemokine Cxcl10 (Cliffe et al., 2005). As described above, IL-4Rα-dependent tuft cell hyperplasia contributes to the tuft cell – ILC2 feedforward loop to amplify type 2 immunity (Gerbe et al., 2016, von Moltke et al., 2016, Howitt et al., 2016). Whether tuft cells also possess any antihelminth effector response is unclear. IL-4Rα-dependent goblet cell hyperplasia and mucus production is a well described feature of the antihelminth type 2 immune response. The increased volume of mucus has been postulated to both trap the luminal worms as well as disrupt their niche (Maizels and Holland, 1998). Indeed, susceptibility to *T. muris* infection is associated with mucus porosity, with resistant mice having a less permeable mucus barrier than susceptible mice (Hasnain et al., 2010). Specific molecules produced by goblet cells have been shown to be directly anthelmintic. As mentioned above, Relmβ is produced by goblet cells following intestinal helminth infection (Artis et al., 2004, Herbert et al., 2009) and has been demonstrated to bind to the tegument of the larval stages of both *T. muris* and the human intestinal helminth *S. stercoralis*, impairing larval chemosensory function and chemotaxis (Artis et al., 2004). In addition, Relmβ is essential in protective immunity to *H. polygyrus* and *N. brasiliensis* (Herbert et al., 2009). Specifically, Relmβ was shown to inhibit the feeding of adult *H. polygyrus* worms *in vivo*, reducing their fitness *ex vivo*, measured by ATP and protein levels (Herbert et al., 2009). Whether this effect on *H. polygyrus* is mediated via disruption of chemosensation or other neuronal processes is unclear. Muc2 and Muc5ac are

mucus components upregulated in response to intestinal helminth infection (Hashimoto et al., 2009, Hasnain et al., 2010, Inagaki-Ohara et al., 2011). Increased Muc2 expression correlated with expulsion of both *T. muris* and *N. brasiliensis,* with Muc2-deficient mice having impaired worm expulsion following *T. muris* infection (Inagaki-Ohara et al., 2011, Hasnain et al., 2010). Despite not normally being expressed in the intestinal tract, Muc5ac expression was detected following *T. muris* infection shortly before worm expulsion, but was not induced upon *H. polygyrus* infection (Hasnain et al., 2011, Hasnain et al., 2010). Muc5ac-deficient mice failed to expel *T. muris* and *N. brasiliensis* (Hasnain et al., 2011). Intriguingly, unlike Muc2, Muc5ac directly impaired the health of *T. muris in vitro*, as measured by parasite ATP levels (Hasnain et al., 2011). This effect was dependent upon the polymerisation of Muc5ac, suggesting that the structure of this mucin is critical to its anthelmintic function.

Enteroendocrine cells are rare in the intestinal epithelium, shown to mediate protection against the intestinal helminth *Trichinella spiralis*. Following infection, $CD4^+$ T cells induced CCK^+ enteroendocrine I cell hyperplasia and CCK hypersecretion, which in turn reduced leptin levels, an inflammatory adipokine. The reduced leptin levels heightened the protective type 2 immune response, whereas exogenous administration of leptin perturbed Th2 cytokine production and mastocytosis, abrogating parasite expulsion (Worthington et al., 2013).

Taken together, these data indicate that epithelial cells are essential in protective immunity to intestinal helminth infection, by both initiating the appropriate immune response and producing key effector molecules.

1.2.3.2 Smooth muscle

The GI tract is made up of a series of organs that process ingested food, assimilate water and nutrients, and eliminate waste. Critical to moving the contents through the intestine are the smooth muscle tissues, *tunica muscularis*, via peristalsis. Smooth muscle cells are autonomous, generating spontaneous electrical rhythmicity and contractions, and form both electrical and mechanical junctions with neighbouring cells to coordinate intestinal motility (Sanders et al., 2012).

Type 2 cytokines have been demonstrated to modulate intestinal contractility (Zhao et al., 2003). Both *H. polygyrus* and *N. brasiliensis* infection increased intestinal hypercontractility *ex vivo*, which was dependent upon IL-4Rα-activated Stat6 signalling, Protease-activated receptor 2 (*Par2*) expression and enteric nerve stimulation (Zhao et al., 2003, Shea-Donohue et al., 2010). Similarly, *T, muris* infection induced increased smooth muscle hypercontractility in resistant strains, however hypercontractility was instead dependent upon IL-9 and not Stat6 signalling (Khan et al., 2003). Administration of an immunosuppressive steroid could partially restore smooth muscle hypercontractility in susceptible mice (Motomura et al., 2010), further demonstrating that the immune system can mediate smooth muscle cell function. Induction of smooth muscle hypercontractility was also shown to be essential for expulsion of both *N. brasiliensis* and *T. muris* (Khan et al., 2003, Horsnell et al., 2007) however the role of smooth muscle contractility in *H. polygyrus* infection has not been determined.

1.3 MicroRNAs

MicroRNAs (miRNAs) are noncoding RNA molecules of approximately 21 nucleotides (nt) in length which function to regulate messenger RNA (mRNA) translation (Ambros, 2003). miRNAs were first discovered in 1993 with the identification of *lin-4* in *Caenorhabditis elegans*. The small, noncoding RNA *lin-4* was identified to negatively regulate *lin-14* mRNA translation into protein through a RNA-RNA interaction (Lee et al., 1993). It was not until 2001 that further miRNA species were identified and demonstrated to be conserved from *C. elegans* to mammals (Lee and Ambros, 2001, Lagos-Quintana et al., 2001, Lau et al., 2001). Over the past 16 years our understanding of miRNA biogenesis and function has improved dramatically, allowing us to further appreciate their importance in many different biological settings.

1.3.1 Biogenesis

Both canonical and non-canonical pathways of mature, functional miRNA molecule biogenesis have been described. Canonical biogenesis consists of several stages, each requiring key enzymes, before producing mature miRNAs. Initially, primary miRNA (pri-miRNA) molecules are transcribed from their respective gene by RNA polymerase II (Lee et al., 2004, Lee et al., 2002). The pri-miRNA molecule has an imperfect self-complementary region, forming a hairpin loop, with elongated 3' and 5' tails. In some cases, several miRNA loci are in close proximity, creating polycistronic miRNA genes or clusters, and are transcribed as one cluster containing many pri-miRNAs (Lee et al., 2002). The pri-miRNA molecule remains in the nucleus where the RNA binding protein DiGeorge syndrome critical region gene 8 (Dgcr8) recognises its structure and recruits the RNase enzyme Drosha. Drosha cleaves the elongated 5' and 3' tails to form a precursor miRNA (pre-miRNA) molecule of approximately 70-nucleotides (nt), with a 2-nt 3' overhang (Lee et al., 2003, Han et al., 2004, Denli et al., 2004, Gregory et al., 2004, Landthaler et al., 2004). This 3' overhang is essential for recognition and binding of the high-resolution nuclear export factor Exportin-5. Exportin-5 facilitates the transport of the pre-miRNA out of the nucleus and into the cytoplasm via a RAN-GTP-dependent mechanism (Yi et al., 2003). In the cytoplasm, the double stranded RNA binding protein TAR RNA-binding protein (TRBP), in complex with the RNase enzyme Dicer, binds and cleaves the hairpin loop of the pre-miRNA to produce the mature, double stranded miRNA molecule (Hutvagner et al., 2001, Ketting et al., 2001, Knight and Bass, 2001). The passenger strand (also known as miRNA*) dissociates from the complementary, mature guide miRNA strand after being recognised, and subsequently bound, by an Argonaute (Ago) protein (Chendrimada et al., 2005). In most instances, the mature strand is that which is most thermodynamically stable and the released passenger strand is degraded quickly (Khvorova et al., 2003, Schwarz et al., 2003). This results in an abundance bias toward the mature guide strand, although the passenger strand is still active and can be bound by Ago proteins. The miRNA-Ago complex is then incorporated into the RNA-induced silencing complex (RISC). In the RISC, the miRNA functions by guiding the complex to complementary sequences in the 3' untranslatable region (UTR) of target messenger RNA to induce gene silencing (Maniataki and Mourelatos, 2005, MacRae et al., 2005, Gregory et al., 2005,

Martinez et al., 2002). The canonical miRNA biogenesis pathway is displayed in **Figure 1.3**. It is now clear that canonical miRNA processing cannot occur without Drosha, Dgcr8 and Dicer.

Non-canonical miRNA biogenesis deviates from the canonical pathway by one or more steps. Dicer however is almost always indispensable in the generation of mature, functional miRNAs in both canonical and non-canonical pathways. Drosha and Dgcr8 are specific to canonical miRNA biogenesis, and therefore in their absence only non-canonical miRNA biogenesis can occur (Abdelfattah et al., 2014).

One such Drosha/Dgcr8 independent non-canonical pathway is the mirtron pathway. This pathway mirrors the canonical in the cytoplasm (utilises Dicer) but does not require the Drosha/Dgcr complex in the nucleus. Pre-miRNA-sized short introns were discovered by deep sequencing of small RNAs, which are processed by spliceosomes and debranching enzymes within the nucleus to produce pre-miRNA-like hairpins suitable for Dicer cleavage (Ruby et al., 2007, Okamura et al., 2007). These pre-miRNA-like hairpins are transported to the nucleus by Exportin-5. Mirtron-derived miRNAs have been found in Drosha- or Dgcr8-deficient mice (Babiarz et al., 2008, Chong et al., 2010).

As well as mirtrons, other Dicer-dependent, non-canonical miRNA biogenesis pathways have been discovered but not fully elucidated. These include miRNAs from endogenous short hairpin RNAs (Babiarz et al., 2008) and transfer RNAs (tRNAs) (Cole et al., 2009). Both of these pathways generate miRNA hairpins that were able to be recognised and cleaved by Dicer to form mature miRNAs.

Both the canonical and non-canonical miRNA biogenesis pathways highlight the importance of Dicer to produce functional, mature miRNA molecules. As a result, genetic deletion of Dicer results in embryonic lethality (Bernstein et al., 2003), highlighting the critical role played by miRNAs. Despite Dicer's importance in miRNA processing, one miRNA has been discovered to be Drosha/Dgcr8 complex-dependent and Dicer-independent (Cheloufi et al., 2010, Cifuentes et al., 2010). Pri-miR-451 is cleaved in the nucleus by Drosha/Dgcr8 to generate an 18bp pre-miRNA. Upon export to the cytoplasm this pre-miRNA is too short to act as a substrate for Dicer, therefore Ago2 mediates the cleavage of pre-miR-451 to be loaded into the RISC (Cheloufi et al., 2010, Cifuentes et al., 2010).

1.3.2 Mechanism of action

As described in section **1.3.1**, miRNAs function as a guide, directing the RISC to the appropriate mRNA to inhibit translation into protein. Specifically, nucleotides 2-7 of the miRNA, referred to as the seed sequence, is crucial for target recognition, determining the binding to the 3' UTR of mRNA (Krol et al., 2010). The downstream nucleotides are less important, but also contribute to base pairing and binding to target mRNAs (Ha and Kim, 2014). miRNAs are able to prevent mRNA translation into protein by both inducing degradation of mRNA and by inhibiting ribosomal translation (Eulalio et al., 2008). In mammals, there are four Ago proteins (Ago1-4). mRNA degradation was thought to be dependent upon Ago2, as Ago2 was the only Ago protein with catalytic slicer activity, whereas Argonaute1, 3 & 4 were thought to inhibit mRNA translation without mRNA degradation (Liu et al., 2004a, Meister et al., 2004). Recently, slicer activity of Ago3 was described (Park et al., 2017).

In humans, all four Ago proteins bind to miRNAs and have no discernible difference in their miRNA repertoire (Dueck et al., 2012), whether this is the case in other cells types and species is currently unclear.

1.3.3 miRNA-mediated regulation of immune responses to intestinal helminth infection

The ability of miRNAs to provide finite control over gene expression implicates them in almost every biological setting; from embryogenesis, physiological development to pathology. Regulation of the immune response requires coordinated control of specific gene expression in participating immune and stromal cells. As such, miRNAs have been demonstrated to be essential in many aspects of immunology and immunity to infection.

Our current understanding of the role of miRNAs in regulating antihelminth immunity draws from studies investigating the type 2 immune response to intestinal helminths and other type 2 immune-driven diseases, such as allergic asthma.

1.3.3.1 miRNA-mediated regulation of adaptive immunity

As described in section **1.2.1**, the adaptive immune system is essential for the clearance of chronic intestinal helminth infection (Katona et al., 1991, Urban et al., 1991a, Koyama et al., 1995, Else and Grencis, 1996). As outlined in section **1.2.1.1**, Th2 cells and their type 2 cytokines promote resistance against intestinal helminth infection. Aberrant $CD4^+$ T cell polarisation and cytokine production results in an ineffective immune response to intestinal helminth infection, best highlighted in immunity to *T. muris* (Bancroft et al., 1994). miRNAs regulate T cell polarisation and cytokine production. Specifically, Dicer-deficient T cells have a preference for the Th1 lineage, even under Th2 polarising conditions, as well as regulating proliferation and survival *in vivo* (Muljo et al., 2005, Cobb et al., 2005). Unsurprisingly, mice with Dicer-deficient T cells are fully susceptible to both *H. polygyrus* and *T. muris* infection as they fail to mount a protective type 2 immune response (unpublished observations, Wilson Lab).

Further investigation of the miRNA profiles of $CD4^+$ T cells has identified individual miRNAs enriched and essential for specific T helper subtype function (Baumjohann and Ansel, 2013). Studies investigating the miRNA transcriptomic landscape of $CD4^+$ T helper cells identified a suite of miRNAs enriched in Th2 cells from both intestinal infection and allergic disease models. Interestingly, the Th2 cells isolated from intestinal helminth infection exhibited many similarities and distinct differences in their miRNAome when compared to Th2 cells derived from house dust mite-challenged mice or *in vitro* polarised Th2 cells (Okoye et al., 2014). This highlights the heterogeneity of Th2 cells, potentially determined by anatomical location or disease setting. miR-155 expression was enriched in Th2 cells and was critical for protective immunity to *H. polygyrus* (Okoye et al., 2014). The authors demonstrate that miR-155 regulated the expression of *S1pr1* (which encodes the protein Sphingosine-1-phosphate receptor 1) in Th2 cells, with *S1pr1* downregulation previously demonstrated to be required for egress of lymphocytes from lymphoid tissue (Rivera et al., 2008).

Many other T cell-intrinsic miRNAs influence T helper cell differentiation during type 2 immune-mediated diseases. miR-21-deficiency promoted Th1 differentiation in a mouse model of allergic airway inflammation, leading to increased IFNγ levels and

reduced eosinophilia (Lu et al., 2011). Furthermore, miR-182 targeting of *RUNX3* in human $CD4^+$ T cells regulated Th1/Th2 differentiation, where overexpression of miR-138 promoted Th2 differentiation and miR-138 inhibition induced Th1-related cytokines and transcription factor expression (Fu et al., 2015). miRNA clusters have also been shown to regulate T helper cell differentiation. Specifically, overexpression of the miRNA cluster miR-23~27~24 negatively impacts the differentiation of Th1, Th2, Th17 and iTreg lineages *in vitro* (Cho et al., 2016). Deletion of the miR-23~27~24 cluster promoted Th2 cell differentiation and IL-4 expression *in vitro* and exacerbated eosinophilic airway inflammation and pathology *in vivo* (Cho et al., 2016, Pua et al., 2016). Mechanistically, both miR-24 and miR-27 have been demonstrated to target a network of genes which regulate IL-4 production, such as *Gata3*, *Cnot6* and *Ikzf1* (Pua et al., 2016). Increased expression miR-19a, a member of the miR-17~92 cluster, was identified in both human and mouse Th2 cells, essential for promoting Th2 differentiation through the inhibition of *Pten*, *Socs1* and *A20* (Simpson et al., 2014). These observations suggest that miR-182, the miR-23~27~24 cluster and/or the miR-17~92 cluster maybe implicated in the development and function of Th2 cells, critical for protective immunity to intestinal helminth infections.

Tregs are a subset of $CD4^+$T cells that are induced upon intestinal helminth infection which function as immunomodulatory cells, suppressing immunity and protecting against immune-mediated pathology (Abbas et al., 2013, Finney et al., 2007, Grainger et al., 2010) but at the cost of promoting chronic infection. miRNAs have been described to contribute to many aspects of Treg biology, from development and differentiation to function. Dicer or Drosha deletion, which abrogated miRNA biogenesis, compromises Treg maintenance and survival *in vivo* (Liston et al., 2008, Zhou et al., 2008, Chong et al., 2008). Despite many miRNAs being reported to contribute to Treg differentiation, survival and function (Li et al., 2015a, Li et al., 2015b, Li et al., 2014, van der Geest et al., 2015, Pan et al., 2015, Zhou et al., 2015, Jiang et al., 2011, Rouas et al., 2009, Singh et al., 2015, Seddiki et al., 2012, Lu et al., 2010b, Lu et al., 2009), very few studies have investigated the role of miRNAs in Tregs in type 2 immunity, such as during intestinal helminth infection. In one such study, the authors observed miR-182 expression was elevated in Tregs isolated from helminth infected mice and that IL-4 increased miR-182 expression in Tregs *in vitro*

(Kelada et al., 2013). Interestingly, miR-182 expression was required in Tregs for suppression of Th2-mediated immune responses and not Th1 responses (Kelada et al., 2013). This suggests that distinct miRNAs maybe important in regulating the function of specific Treg subsets.

B cells contribute to protection against intestinal helminth infection (Blackwell and Else, 2001, Liu et al., 2010, Wojciechowski et al., 2009, McCoy et al., 2008) and underpin the hopes in the development of an antihelminth vaccine. miR-155 and miR-182 are both essential for the generation of antigen specific, high affinity IgG1 antibody production (Li et al., 2016, Vigorito et al., 2007, Thai et al., 2007), essential for immunity to *H. polygyrus* (McCoy et al., 2008). Specifically, miR-155-deficient B cells have impaired germinal centre and extra-follicular responses following immunisation and therefore failed to produce high affinity IgG1 antibodies (Vigorito et al., 2007, Thai et al., 2007). Later studies identified that miR-155 regulates B cell survival and function, with miR-155 targeting *Jarid2*, preventing B cell apoptosis (Nakagawa et al., 2016), and activation-induced cytidine deaminase (AID), essential for antibody affinity maturation (Teng et al., 2008). Although miR-182 deficiency did not affect B cell development, they exhibited impaired early generation of antigen-specific IgM and IgG1 following immunisation, as well as a reduction of extra-follicular plasma cells (Li et al., 2016). In addition to regulating antibody production, miRNAs are also essential for B cell survival (Tsai et al., 2015). For example, miR-148 is the most abundantly expressed miRNA in plasma cells and critical for their differentiation and survival by targeting both *Bach2* and *Mitf*, which repress Blimp-1 and Irf4 (Porstner et al., 2015). Furthermore, IL-6 and stromal cell-derived factor 1 induced plasma cell survival is dependent upon induction of miR-24-3p (Gabler et al., 2013), potentially by targeting *Bim* (Nguyen et al., 2013). Taken together, miRNAs are essential for regulating B cell survival and antibody generation, although the precise role of B cell-intrinsic miRNAs has not been investigated in the context of antihelminth immunity.

1.3.3.2 miRNA-mediated regulation of innate immunity

Many innate immune cells are essential for protective immunity to intestinal helminth infection, contributing to the initiation of the type 2 immune response and acting as critical effector cells (see section **1.2.2**).

Macrophages are essential effector cells in mediating protection against intestinal helminth infection, outlined in section **1.2.2.2**. Specifically, the alternate activation of macrophages is critical for immunity to *H. polygyrus* (Anthony et al., 2006). The IL-4Rα-Stat6 signalling axis induces a miRNA signature that is conserved between mouse and human (Czimmerer et al., 2016), of which regulates the activation and function of aaMϕs. Specifically, macrophage-intrinsic expression of miR-223 is a critical regulator of macrophage polarisation. miR-223 inhibition of *Pknox1* translation suppressed classical activation (or M1 polarisation) and promoted alternate activation (Zhuang et al., 2012). Furthermore, mir-342-3p acts as a brake following alternate activation of macrophages, limiting aaMϕ survival by inhibiting an anti-apoptotic gene signalling network (Czimmerer et al., 2016). Likewise, miR-378-3p functioned to limit excessive alternate activation and proliferation of macrophages by limiting the IL-4Rα/PI3K/Akt-signalling cascade through the targeting of *Akt1* (Ruckerl et al., 2012). One caveat of recent efforts to investigate the role of miRNAs in macrophage polarisation and function is that the majority of studies utilised *in vitro* model systems. It is therefore imperative to translate these findings *in vivo* to determine if these miRNA regulatory nodes are maintained in a complex, multicellular environment. Overall, with specific miRNAs demonstrated to both promote and limit aaMϕs, we would predict that these macrophage-intrinsic miRNAs maybe essential for immunity to intestinal helminth infections.

DCs are also essential for T cell differentiation and for reactivation of the adaptive immune system (Banchereau and Steinman, 1998, Kapsenberg, 2003) (see section **1.2.2.3**). DC function is also regulated by miRNAs. For example, increased miR-23b expression in DCs prompted a tolerogenic phenotype through the inhibition of Notch1/NF-ϰB signalling pathways, resulting in an inhibition of IL-12 production, increased IL-10 production and an enhanced capability to induce Treg differentiation (Zheng et al., 2012). DC-intrinsic miR-155 expression is essential for their ability to promote Th2 differentiation. Specifically, miR-155 regulates DC-mediated activation

of antigen-specific T cell responses through the targeting of *Arg2* expression. Increased *Arg2* prevented T cell activation and proliferation due to Arginase-2-mediated arginine depletion in the extracellular milieu (Dunand-Sauthier et al., 2014). Furthermore, miR-155 regulates purinergic receptor signalling in DCs that are required for appropriate DC activation and DC-mediated Th2 cell differentiation (Zech et al., 2015). In corroboration with these findings, miR-155-deificent mice fail to expel a 2º *H. polygyrus* infection due to a defective type 2 immune response (Okoye et al., 2014). miR-106b also regulates DC function, preventing Th2 cell polarisation through inhibition of *Egr2* (Tang et al., 2015). Interestingly, the cestode *Taenia crassiceps* can alter DC function through modulation of host miRNAs. *T. crassiceps* ES products regulated LPS-induced miRNA expression profile in human DCs, inhibiting LPS-induced Let-7i expression to reduce DC inflammatory cytokine production (Terrazas et al., 2013). Overall, at least two miRNAs, miR-155 and miR-106b, have been identified to effect DC-mediated Th2 polarisation with other miRNAs regulating tolerogenic DC properties. It is therefore likely that miRNAs are critical in shaping DC-mediated antihelminth immunity.

Mast cells play an important role in antihelminth immunity, contributing to early cytokine release and immune response initiation, as well as the production of inflammatory mediators (see section **1.2.2.4**). miRNAs are essential for mast cell development *in vivo*, with mice harbouring a mast cell-specific Dicer-deletion having a profound mast cell depletion in all tissues (Oh et al., 2014). Upon activation of mast cells, their miRNA expression profile changes (Teng et al., 2015), suggesting that miRNAs mediate mast cell function. Indeed, miR-155 expression regulated mast cell function following activation by targeting components of the PI3K pathway. miR-155-deficient mast cells had enhanced FcεRI-mediated degranulation and release of TNFα, IL-13, and IL-6 (Biethahn et al., 2014). Furthermore, mast cell-intrinsic miR-223 expression regulates IL-6 secretion through targeting *Akt* and *Igf1* (Yang et al., 2016). Expression of miR-221-3p in mast cells promotes the production of IL-4 by modulating the NF-ϰB signalling pathway by directly targeting *Pten* (Zhou et al., 2016). With IL-4 essential for immunity to intestinal helminth infection (Urban et al., 1991b, Bancroft et al., 2000, Bancroft et al., 1998), perhaps increasing IL-4 production from mast cells through miRNA manipulation can promote antihelminth immunity. Overall, with miRNAs regulating a plethora of mast cell functions, it is

therefore likely that mast cell-intrinsic miRNAs contribute to mast cell-mediated antihelminth immunity.

With the large secretion of protein mediators from ILCs, it is interesting to speculate that miRNAs are rapidly downregulated upon activation to permit such high rate if mRNA translation, ribosome activity and protein secretion. As yet, the role of miRNAs in ILCs remains an area of research currently understudied. As outlined in section **1.2.2.1**, ILC2s are important players in antihelminth immunity, secreting type 2 cytokines IL-4, IL-5 and IL-13 to promote type 2 immunity (Moro et al., 2010, Neill et al., 2010, Pelly et al., 2016). Currently, the only miRNA studied in ILC2s is miR-155, with expression of miR-155 in ILC2s increased following activation and required for both proliferation and cytokine production (Johansson et al., 2017). Despite the exact mechanism of miR-155-mediated ILC2 inflammation being unclear, miR-155-deficient ILC2s expressed less *Gata3* and produced less IL-13 (Johansson et al., 2017). Identifying other miRNAs that regulate ILC2 proliferation and type 2 cytokine secretion will be beneficial in further understanding their role in antihelminth immunity.

1.3.3.3 miRNA-mediated regulation of stromal immunity

As outlined in section **1.2.3**, stromal cells are essential for antihelminth immunity. Epithelial cells are particularly important, detecting and initiating immune responses to intestinal helminths as well as acting as important effector cells (see section **1.2.3.1**). Genetic deletion of the miRNA processing enzyme Dicer in intestinal epithelial cells resulted in the disorganisation of intestinal architecture, reducing goblet cell numbers and decreasing barrier function (McKenna et al., 2010). Unsurprisingly, mice with Dicer-deficient IECs are susceptible to *T. muris*, unlike their WT counterparts, due to a failure to induce Relmβ expression and induce a futile Th1 immune response (Biton et al., 2011). In WT mice, IL-13 signalling in IECs drives miR-375 expression, concomitant with an increase in TSLP mRNA and protein expression. miR-375-deficient mice partially phenocopied mice with Dicer-deficient IECs, with reduced goblet cell frequency and reduced expression of Relmβ and TSLP mRNA in the small intestine (Biton et al., 2011). Although the targets of miR-

375 were not identified in this study, it is likely that miR-375 targets an inhibitor of IL-13R-mediated signalling and that deletion of miR-375 allows for increased expression of the inhibitory pathway, thus increasing expression of TSLP. miR-375 also negatively regulates enteroendocrine cell development (Knudsen et al., 2015), an IEC subtype critical for protection against *T. spiralis* (Worthington et al., 2013). Furthermore, patients with ulcerative colitis have reduced miR-375 expression (Wu et al., 2008), adding to the growing evidence that miR-375 may be an important miRNA regulating intestinal epithelial cell homeostasis and potentially in regulating immunity to intestinal helminth infections.

Tuft cells are another subtype of IEC, which specialise in the detection of intestinal helminth and the subsequent initiation of type 2 immunity through release of IL-25 (Gerbe et al., 2016, von Moltke et al., 2016, Howitt et al., 2016). Whether miRNAs are important for tuft cell differentiation and function is currently unclear. However, miR-20b has been demonstrated to directly target IL-25, with an inverse relationship between serum levels of miR-20b and IL-25 observed in myasthenia gravis patients (Chunjie et al., 2015). Furthermore, elevated levels of miR-20b have been observed in the colon of patients with ulcerative colitis (Coskun et al., 2013), who have previously been described to have reduced intestinal IL-25 (Caruso et al., 2009). The role of miR-20b has not been investigated in the context of antihelminth immunity, however, extrapolating previous research, reducing miR-20b expression may allow for increased IL-25 production and therefore enhance antihelminth immunity.

Intestinal microfold cells (M cells) are a specialised IEC, which are located on the luminal surface of gut-associated lymphoid tissue (GALT). M cells are important transporters of luminal antigens from the intestinal lumen into the GALT and are likely to be important in the generation of adaptive immune responses following helminth infection, but have not yet been directly studied in this context. M cell maturation relies on functional miRNA expression, with Dicer deletion restricted to IECs significantly depleted of M cell numbers and impairing antigen uptake (Nakato et al., 2016).

The critical role of Dicer in epithelial cell biology, coupled with the essential functions of IECs in regulating intestinal homeostasis and antihelminth immunity, strongly suggest a role for IEC-intrinsic miRNAs in mediating protection against intestinal

helminth infection. The precise function of specific IEC-intrinsic miRNAs is currently understudied, but investigation could elucidate critical IEC genes and pathways in antihelminth immunity.

1.3.3.4 Helminth-derived miRNAs

Intestinal helminths have long been known to produce and secrete molecules and proteins that act on the host to influence immunity. Many of these molecules induce an immunoregulatory environment, dampening the host immune response, preventing immunopathology and promote chronic infection.

More recently, intestinal helminths have been shown to produce miRNAs which are incorporated and act in host cells. Specifically, Buck and colleagues identified miRNA-filled exosomes that were secreted by *H. polygyrus*, which contain a miRNA profile distinct from that isolated form the total worm (Buck et al., 2014). Administration of *H. polygyrus*-derived exosomes to mice was able to supress type 2 responses by targeting *Il33r* and *Dusp1*. This study suggests intestinal helminths may select particular miRNAs for secretion to contribute to their immunoregulatory effector properties. In addition, miRNAs were detected in exosome-like vesicles in the ES products of *T. muris*, although their ability to act on the host is unclear (Tritten et al., 2017). Furthermore, parasite-derived miRNAs have been found in the circulation of the host. For example, miRNAs were found in the host blood following infection with the filarial nematodes *Litomosoides sigmodontis* (mice), *Onchocerca volvulus* (human), *Onchorcerca ochengi* (cows), *Loa loa* (baboons) and *Dirofilaria immitis* (Dogs) (Buck et al., 2014, Tritten et al., 2014b, Tritten et al., 2014a). Whether these filarial nematode-derived miRNAs can target host mRNAs is unclear. However, it is interesting to speculate that parasite-derived miRNAs can modulate host immunity and influence susceptibility and chronicity of infection.

1.4 Phospholipase A_2 (PLA_2)

1.4.1 PLA_2 function and mechanism

Phospholipase A_2 (PLA_2) enzymes are a large superfamily of enzymes consisting of 5 distinct types, 15 groups and multiple isoforms. PLA_2 enzymes hydrolyse fatty acid moieties from the *sn*-2 position of phospholipids, yielding nonesterified (unsaturated) fatty acids and lysophospholipids (**Figure 1.4**) (Leslie, 2004, Berg et al., 2001, Six and Dennis, 2000). Both the fatty acids and lysophospholipids produced lead to the induction of several downstream responses. The fatty acids released, such as arachidonic acid, can be metabolised to form various eicosanoids and other bioactive lipids and mediate a wide range of biological functions (Powell, 2005). Lysophospholipids, such as lysophosphatidylcholine (LPC), are bioactive, capable of inducing distinct biological effects (Kabarowski, 2009, Valentine et al., 2008). As a result, PLA_2 enzymes contribute to a diverse array of biological events.

1.4.2 PLA_2 family members

As mentioned above, the superfamily of PLA_2 enzymes compasses 15 different groups that fall into 5 distinct types. PLA_2 enzymes are separated into types based on their function, including secretory PLA_2s, cytosolic PLA_2s, calcium-independent PLA_2s, lysosomal PLA_2s and lipoprotein-associated PLA_2s. Each PLA_2 enzyme is assigned a group (I – XV), which is determined by their structure and characteristics. Each individual PLA_2 isoform is then identified using a letter after the assigned group, for example PLA_2 group IIA (PLA_2g2A). The precise function of PLA_2 varies between each individual member, often depending where they are expressed and their cellular location. Each family type is outlined below.

1.4.2.1 Secretory PLA_2s ($sPLA_2$s)

Secretory PLA_2s ($sPLA_2$s) were the first PLA_2 enzymes to be identified. PLA_2s enzymes were first discovered in snake venoms and characterised after the purification from both cobra and rattlesnake venom in the 1970's. The two venoms

phospholipase enzymes have a large number of cysteines in the form of disulphide bonds, with six disulphide bonds in common but one bond located in a distinctly different location. These two venom PLA_2s were designated as group I (cobra venom PLA_2) and group II (rattlesnake venom PLA_2) (Dennis et al., 2011). At the same time, studies on the pancreatic digestive enzymes identified a mammalian $sPLA_2$ enzyme, with the same disulphide bonding pattern as that seen in the cobra venom. This $sPLA_2$ was therefore designated as group IB. Another $sPLA_2$ was discovered in the synovial fluids of arthritic joints which shared the disulphide bonding characteristics of the rattlesnake venom, therefore this $sPLA_2$ was denoted as group IIA (Hara et al., 1989). A $sPLA_2$ purified from bee venom was named as a group III PLA_2 due to further differences in its structure (Kuchler et al., 1989). A further $sPLA_2$ was identified in macrophages, however this $sPLA_2$ lacked a seventh disulphide bond and was subsequently named as a group V PLA_2 (Dennis et al., 2011). More $sPLA_2$ enzymes have since been discovered, each with differing structures or disulphide bonding characteristics, giving rise to groups IX, X, XI, XII, XIII and XIV (Dennis et al., 2011, Murakami et al., 2015).

Structurally, $sPLA_2$s enzymes are closely related low-molecular weight molecules, with highly conserved calcium binding loop and conserved disulphide bonds. Each $sPLA_2$ isoform has been demonstrated to distinct substrate specificity, determined by the polar head groups of the phospholipid (Mitsuishi et al., 2007). Many of the functions of $sPLA_2$ enzymes have been identified using both knockout and overexpressing mice. Their functions are varied and differ between isoforms, such as membrane remodelling, modification of dietary lipids and degradation of microbe phospholipids. This breadth of functions highlights the distinct roles of each $sPLA_2$ isoform *in vivo*, likely due to their subtleties in different enzymatic properties, substrate specificity and tissue distribution.

As well as mediating biological functions through their catalytic activity, $sPLA_2$s can also act as ligands that interact with $sPLA_2$ receptors ($sPLA_2$Rs), independent of catalytic function (Granata et al., 2005, Triggiani et al., 2003, Mandal et al., 2001). Two types of $sPLA_2$R have been described in mammals, the N-type and M-type receptors (Valentin and Lambeau, 2000). The N-type receptor is abundantly expressed in the brain whereas the M-type receptor is expressed in various tissues (Lambeau et al., 1991, Lambeau and Lazdunski, 1999). The M-type receptor exists

as both membrane bound and soluble receptor (Lambeau and Lazdunski, 1999). The membrane bound form can induce signalling cascades upon ligand activation (Granata et al., 2005) whereas the soluble receptor sequesters and clears extracellular $sPLA_2$, demonstrated to be important in limiting inflammation (Tamaru et al., 2013).

1.4.2.2 Calcium-dependent cytosolic PLA_2s ($cPLA_2$s)

In 1992, two groups independently reported the purification and characterisation of the first human cytosolic PLA_2 ($cPLA_2$) from a macrophage cell line, whose sequence was unrelated to $sPLA_2$ enzymes previously described and was therefore denoted as a group IV PLA_2 (Sharp et al., 1991, Clark et al., 1991). There are 6 $cPLA_2$s, all of which are group IV members and dependent upon calcium for their catalytic activity (Leslie, 2015). Each of the 6 isoforms exhibit slightly different properties, sharing only 30% homology (Ohto et al., 2005), specifically in substrate specificity and sensitivity to pharmacological inhibitors (Ghomashchi et al., 2010).

Among the $cPLA_2$ group IV family members, phospholipase A_2 group IVA (PLA_2g4A) is the most well characterised, highly conserved between mouse and human (Clark et al., 1991) and expressed in a wide range of cells throughout the body, such as mast cells, endothelial cells and lung fibroblasts (Nakatani et al., 2000, Ghosh et al., 2004, Tanaka et al., 2011). $cPLA_2$g4A activity is predominantly regulated by its intracellular location. Normally sequestered in the cytosol, translocation to membranes (cell or organelle) is induced by an increase in intracellular calcium ion concentration (Channon and Leslie, 1990, Gijón et al., 1999). Calcium is also essential for functional catalytic activity, although the binding domains of calcium differ to that required for membrane translocation (Xu et al., 1998). PLA_2g4A activation is also dependent upon the calcium-independent binding of the lipid second messenger phosphatidylinositol-4,5-bisphosphate (PIP_2) (Mosior et al., 1998, Six and Dennis, 2003) and phosphorylation (Dennis et al., 2011). PLA_2g4c has a specificity for arachidonic acid and is important for the generation of eicosanoids, such as prostaglandins (PGs) and leukotrienes (LTs), and bioactive lysophospholipids (Leslie, 2004).

Evidence of other roles of cPLA$_2$s have also emerged. PLA$_2$g4A also regulates both Golgi-membrane and intra-Golgi trafficking (San Pietro et al., 2009, Regan-Klapisz et al., 2009). Furthermore, PLA$_2$g4C was discovered in the epithelium of lesions from psoriatic patients, where it is postulated to be involved in the generation of eicosanoid-mediated chronic inflammation and disease (Chiba et al., 2004, Ghosh et al., 2006). Despite the discovery of other cPLA$_2$ isoforms, their biological roles have been difficult to elucidate and are still unknown.

1.4.2.3 Calcium-independent cytosolic PLA$_2$s (iPLA$_2$s)

In the 1994, another PLA$_2$ was identified in the cytosol of macrophage-like cells, however the protein sequence differed from cPLA$_2$s and its enzymatic activity was not dependent on calcium. It was therefore named as a calcium-independent PLA$_2$ (iPLA$_2$) and designated as a group VI PLA$_2$, isoform A (PLA$_2$g6A) (Ackermann et al., 1994, Tang et al., 1997). The iPLA$_2$ family only encompasses group VI PLA$_2$s despite the existence of other calcium-independent PLA$_2$s, such as the lysosomal PLA$_2$s (lPLA$_2$s, section **1.4.2.4**) and the cPLA$_2$ PLA$_2$g4C (section **1.4.2.2**). There are 6 members of the group VI iPLA$_2$ family (A-F), all of which are catalytically active. The catalytic domain of iPLA$_2$ enzymes is also called a patatin-like domain as it shares 40% homology to patatin, another enzyme with lipase activity (Hsu et al., 2009). Although not regulated by calcium, PLA$_2$g6A activity and function is regulated by ATP binding and caspase cleavage. PLA$_2$g6A can be activated by ATP in the mitochondria of various cell types (Liou et al., 2005, Gadd et al., 2006), although ATP is not a substrate, but instead acts as a cofactor for PLA$_2$g6A activity, likely inducing a conformational change (Lio and Dennis, 1998). Caspase proteolysis of the PLA$_2$g6A enzyme during apoptosis results in a hyperactive, truncated protein (Atsumi et al., 2000, Atsumi et al., 1998), resulting in membrane damage and processing of lysophospholipids to initiate phagocytosis (Kim et al., 2002, Lei et al., 2010).

Like cPLA$_2$s, iPLA$_2$s are intracellular enzymes which are ubiquitously expressed and contribute to a variety of biological activities, such as fat catabolism, cell differentiation, mitochondrial maintenance, phospholipid remodelling and signal

transduction (Ramanadham et al., 2015). As expected, dysregulation of iPLA$_2$ enzymes has been linked to a wide range of diseases.

1.4.2.4 Lysosomal PLA$_2$s (lPLA$_2$s)

In 1996, group XV PLA$_2$ (PLA$_2$g15) was discovered in the soluble and lysosomal fractions of Madin-Darby canine kidney (MDCK) cells (Abe et al., 1996) and subsequently identified to be ubiquitously expressed in every tissue (Abe et al., 2004). PLA$_2$g15 is the only member of the lysosomal PLA$_2$ (lPLA$_2$) family, with optimal enzymatic activity at pH 4.5 (Abe et al., 1996). The catalytic activity of PLA$_2$g15 is not dependent upon calcium, but millimolar concentrations of calcium or magnesium can enhance its activity (Abe and Shayman, 1998). Although primarily functioning as a PLA$_2$, PLA$_2$g15 also possesses transacylase activity in acidic conditions (Abe et al., 1996).

Although expressed in every tissue, the specific activity of PLA$_2$g15 was 50 times greater in alveolar macrophages (Abe et al., 2004), suggesting lPLA$_2$ to have an important role in these cells. Indeed, lPLA$_2$ may be involved in surfactant phospholipid catabolism in alveolar macrophages. CSF2-deficient mice develop a progressive accumulation of the phospholipid-rich pulmonary surfactant (Dranoff et al., 1994), correlating with a significantly lower lPLA$_2$ activity and expression than WT (Abe et al., 2004). Moreover, *Pla2g15*-deficient mice have increased lung phospholipid-rich pulmonary surfactant (Hiraoka et al., 2006). *Pla2g15*-deficient mice, backcrossed onto an apo E-null background had increased development of high fat diet-induced atherosclerosis, characterised by increased formation of foamy macrophages (or foam cells) due to defective phospholipid degradation (Taniyama et al., 2005, Hiraoka et al., 2006), suggesting a critical role for lPLA$_2$ in protection against atherosclerosis.

Together, these data suggest that lPLA$_2$ has an essential role in macrophages and maintaining homeostasis of various organs and tissues.

1.4.2.5 Lipoprotein-associated PLA_2s ($lpPLA_2$s)

Lipoprotein-associated PLA_2s ($lpPLA_2$s), also known as platelet activation factor (PAF) acetylhydrolases (PAF-AHs), contain two groups of PLA_2s, group VII and group VIII. As the family name suggests, PLA_2g7A is a secreted enzyme associated with both high- and low-density lipoproteins (HDL and LDL) in human plasma, with LDL-bound PLA_2g7A more active than HDL-bound PLA_2g7A (Stafforini et al., 1989, Stafforini et al., 1987). However, the family name is misleading as the remaining members, PLA_2g7B and PLA_2g8, are both intracellular proteins and not associated with lipoproteins. All three members catalyse the hydrolysis of PAF to produce lyso-PAF and acetate, in a calcium-independent manner (Schaloske and Dennis, 2006, Dennis et al., 2011).

PLA_2g7A is implicated in the development of atherosclerosis and neonatal necrotising enterocolitis. PLA_2g7A is predominantly secreted by macrophages, with expression and secretion significantly increased in macrophages in atherosclerotic lesions (Stafforini et al., 1990, Elstad et al., 1989), however it is unclear whether PLA_2g7A activity is anti- or pro-atherosclerotic. Premature human infants with neonatal necrotising enterocolitis have decreased serum PLA_2g7A levels and increased systemic accumulation of PAF (Caplan et al., 1990). *Pla2g7a*-deficient mice have increased incidence of neonatal necrotising enterocolitis, suggesting that PLA_2g7A may be protective (Lu et al., 2010a). Intracellular PLA_2g7B is predominantly expressed in epithelial cells (Kono et al., 2008) and is thought to have an antioxidant function, protecting the cell against oxidative stress-induced cell death (Matsuzawa et al., 1997). *Pla2g7b*-deficient mice have a delayed recovery following hepatic injury (Kono et al., 2008) and *Pla2g7b*-overexpressing mice are protected against neuronal injury (Umemura et al., 2007). PLA_2g8 is found in the brain as a heterotrimeric protein complex (Ho et al., 1997). Its biological function is unclear, however it does seem to be implicated in brain development (Dennis et al., 2011).

1.4.3 Phospholipase A_2 group 1B (PLA_2g1B)

PLA_2g1B was the first mammalian phospholipase to be discovered, a $sPLA_2$ (see section **1.4.2.**1) closely related to the PLA_2 extracted from cobra venom (Dennis et

al., 2011), and as such is often called an ‘old’ phospholipase. *Pla2g1b* expression is detected in multiple tissues in the mouse with the highest expression detected in organs associated with the gastrointestinal tract. The highest expression is observed in the stomach, pancreas, small intestine and lung, respectively (Eerola et al., 2006). In most cases, the expression of *Pla2g1b* correlates with the PLA_2g1B protein content detected in each organ, with the highest concentration detected in the stomach, pancreas, small intestine and salivary gland (Eerola et al., 2006).

Like other $sPLA_2$, PLA_2g1B is a small enzyme (13-15 kDa), contains 7 disulphide bonds and requires calcium for its catalytic activity (Burke and Dennis, 2009). PLA_2g1B is synthesised as an inactive zymogen, before cleavage of an N-terminal peptide is cleaved by trypsin or plasmin (Kudo and Murakami, 2002, Nakano et al., 1994). *In* vitro, PLA_2g1B has strict fatty acid substrate selectively, but selectivity is affected by the phospholipid head group (Murakami and Kudo, 1997). Specifically, PLA_2g1B most effectively hydrolyses anionic phospholipids, such as phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and phosphatidylserine (PS) over charge-neutral phosphatidylcholine (PC) (Snitko et al., 1999). The binding and hydrolysis of PC can be greatly increased in the presence of a detergent such as deoxycholate (deoxycholic acid is a bile acid) (Hanasaki et al., 1999), important as dietary phospholipid hydrolysis by pancreatic PLA_2g1B occurs in the presence of bile in the small intestine.

PLA_2g1B has predominantly been studied in the context of dietary lipid digestion, with important roles in mediating diet-induced disease. However, expression of *Pla2g1b* in tissues outside the digestive tract suggests other biological functions. Indeed, further functions have been described for PLA_2g1B, including immune cell modulation, however these are less well studied.

1.4.3.1 Role of PLA_2g1B in dietary lipid remodelling

With high expression in the digestive system, the primary function of PLA2g1B is believed to be involved in dietary lipid remodelling to enable absorption of lysophospholipids. Upon feeding, PLA2g1B is released from pancreatic acinar cells granules into the pancreatic juice and transported to the small intestinal lumen where

it can mediate lipid digestion and absorption (Hui, 2012). However, *Pla2g1b*-deficient mice display normal dietary lipid absorption, suggesting that other enzymes can compensate for the lack of PLA_2g1bB (Richmond et al., 2001).

A genome wide association study identified a single nucleotide polymorphism in the human *PLA2G1B* gene as a risk factor for obesity (Wilson et al., 2006). Studies blocking PLA_2g1B identified a critical role for this enzyme in diet-induced obesity. Specifically, PLA_2g1B blockade or genetic deletion protected mice from high fat diet-induced postprandial fat absorption and weight gain (Huggins et al., 2002, Hui et al., 2009). Mechanistically, *Pla2g2b*-deficient mice were found to be resistant to obesity due to their ability to maintain elevated energy expenditure and core body temperature when subjected to a high calorie diet. PLA_2g1B-mediated production and absorption of LPC supressed hepatic fat utilisation and downregulated energy expenditure (Labonte et al., 2010).

Furthermore, *Pla2g1b*-deficient mice were protected from high fat diet-induced insulin resistance (Huggins et al., 2002). This phenotype was later described to be due to a reduction of PLA_2g1B-mediated postprandial lysophosphatidylcholine (LPC) absorption during a high fat diet, which supressed insulin-stimulated glycogen synthesis to control blood glucose concentration (Labonte et al., 2006). This phenotype was also observed with the use of PLA_2g1B-specific inhibitors in WT mice fed a high fat diet (Hui et al., 2009). Moreover, overexpression of *Pla2g1b* in pancreatic acinar cells exacerbated high fat diet-induced obesity and insulin resistance (Cash et al., 2011).

PLA_2g1B also induced hyperlipidemia in mice fed on a hypercaloric diet (high fat and high carbohydrate diet). *Pla2g1b*-deficient mice were protected from elevated plasma triglyceride and cholesterol levels induced by a hypercaloric diet, with protection dependent upon reduced hepatic very low-density lipoprotein (VLDL) production and increased triglyceride-rich lipoprotein clearance (Hollie and Hui, 2011). Given that *Pla2g1b*-deficiency reduced hyperlipidemia, it is no surprise that *Pla2g1b*-deficient mice were also resistant to diet-induced atherosclerosis, when crossed with atherosclerosis prone *Ldlr*-deficient mice (Hollie et al., 2014).

Taken together, PLA_2g1B plays a major role in the induction of diet-induced diseases through dietary lipid remodelling and subsequent absorption. Therefore,

pharmacological inhibition of PLA_2g1B maybe of therapeutic use to treat diet induced obesity and associated diseases, such as diabetes and atherosclerosis.

1.4.3.2 Role of PLA_2g1B in immunity

As described above in section **1.4.3.1**, much of the current research into the biological roles and functions of PLA_2g1B has been focussed toward dietary lipid remodelling, and subsequent effects on diet-induced diseases, with very little focus on immunology.

Other members of the $sPLA_2$ family (see section **1.4.2.1**) have been implicated in immune responses, infection and inflammatory disease (reviewed in (Murakami et al., 2016)). For example, PLA_2g3 drives mast cell maturation in a paracrine manner (Taketomi et al., 2013), PLA_2g10 is released from airway epithelial cells upon allergen exposure to induce leukotriene secretion by eosinophils (Henderson et al., 2007, Lai et al., 2010) and other members contribute to antifungal and antibacterial defence (Balestrieri et al., 2009, Weinrauch et al., 1998). The precise mechanisms by which $sPLA_2$s mediate their effects on the immune system is not clear. This, in part, is due to the dual functions of the protein- via catalytic activity or via binding to its receptor. Receptor binding can induce different responses depending on the cell type and the soluble M-type PLA_2R can act as a sponge, sequestering excess $sPLA_2$ and preventing the catalytic activity. Also, $sPLA_2$ enzymatic activity produces many bioactive products with direct biological activity or indirect actions via downstream pathways.

PLA_2g1B has various effects on immune cells *in vitro*. Stimulation of human neutrophils with PLA_2g1B induced the production of the chemokine CXCL8(Jo et al., 2004) and LTB_4 (Lee et al., 2005). Interestingly, PLA_2g1B does not directly induce extracellular arachidonic acid release, but instead acted via the $sPLA_2$R to induce both activation of the MAPK-ERK signalling pathway or $cPLA_2$ activation and eicosanoid production (Mandal et al., 2001, Jo et al., 2004, Lee et al., 2005). PLA_2g1B also exerted pro-apoptotic effects on a mouse macrophage cell line, again via $sPLA_2$R binding and production of TNFα (Lee et al., 2006), as well as proliferative and migratory effects on fibroblasts (Arita et al., 1991). It is unlikely that these effects

are specific to PLA_2g1B, but similar effects are induced by all $sPLA_2$ family members, as the $sPLA_2$ receptor binds all other $sPLA_2$ members.

PLA_2g1B is also implicated in mediating lung inflammation. Indeed, PLA_2g1B induced TNFα and IL-6 production from primary human lung macrophages in a concentration dependent manner. Cytokine production was not dependent on the catalytic activity and instead relied upon the M-type $sPLA_2$R, inducing ERK1/2 phosphorylation (Granata et al., 2005). In addition, deletion of the $sPLA_2$R exacerbated allergic lung inflammation in mice, reducing clearance of PLA_2g1B (Tamaru et al., 2013). Increased PLA_2g1B concentrations correlated with increased eicosanoid and T_H2 cytokine concentration as well as an increased infiltration of eosinophils and neutrophils (Tamaru et al., 2013). This study suggests that in the lung, $sPLA_2$R acts as a sponge to sequester excess PLA_2g1B and limit its activity. This is likely to be mediated by the soluble $sPLA_2$R, although has yet to be determined.

Overall, like other $sPLA_2$s, PLA_2g1B has the potential to influence immunity by affecting multiple cell types. However, the role of $sPLA_2$s enzymes, including PLA_2g1B, in intestinal helminth infection has not been studied.

1.5 Thesis aims

Intestinal helminth infections are highly prevalent, infecting approximately a third of the world's human population as well as a significant number of livestock. As a result, intestinal helminth infections are responsible for significant health and economic burdens (Bartsch et al., 2016, Fitzpatrick, 2013, Bethony et al., 2006). With a small number of chemotherapeutic drugs available, evidence of drug-resistant helminths (Kaplan and Vidyashankar, 2012) and stalling vaccine efforts, it is imperative to improve our understanding of antihelminth immunity to identify new therapeutic avenues.

Our current understanding of antihelminth immunity places type 2 immunity at the forefront, with type 2 cytokines mediating various expulsion mechanisms (Grencis, 2015, Allen and Maizels, 2011). However, the mechanism of helminth killing prior to expulsion remains unclear.

In this thesis, I aim to identify novel mechanisms of antihelminth immunity. To address this, we utilised the mouse model of STH, *H. polygyrus*. We used this parasite as it is a naturally occurring intestinal helminth of the mouse, in which C57BL/6 mice are susceptible to a 1^{o} infection, establishing a chronic infection (Reynolds et al., 2012). However, following drug-cure of 1^{o} infection, C57BL/6 mice are resistant to 2^{o} challenge infection (Finkelman et al., 1997). Using this model system allowed us to interrogate features associated with susceptibility and resistance in the same genetic background. We performed both mRNA- and miRNA sequencing of small intestinal tissue from both susceptible and resistant mice to identify transcriptional changes at the site of infection.

Using the mRNA sequencing data, we identified novel genes and pathways that we hypothesised to contribute to resistance to intestinal helminth infection (**Chapter 3**). Furthermore, using miRNA sequencing, we also investigated the role of miRNAs in regulating protective immunity to *H. polygyrus*. miRNA-mediated coordinated control of gene expression has been shown to be essential in infection and immunity, promoting and fine-tuning the appropriate immune response. Despite this, the role of miRNAs in antihelminth immunity is relatively understudied. We hypothesise that miRNAs are differentially expressed between susceptible and resistant mice and therefore regulate protective immunity to *H. polygyrus* (**Chapter 4**).

By studying both mRNA and miRNA transcriptional changes in mice susceptible and resistant to *H. polygyrus*, we aim to uncover novel genes and pathways critically required for protective immunity to intestinal helminth infection.

1.6 Figures

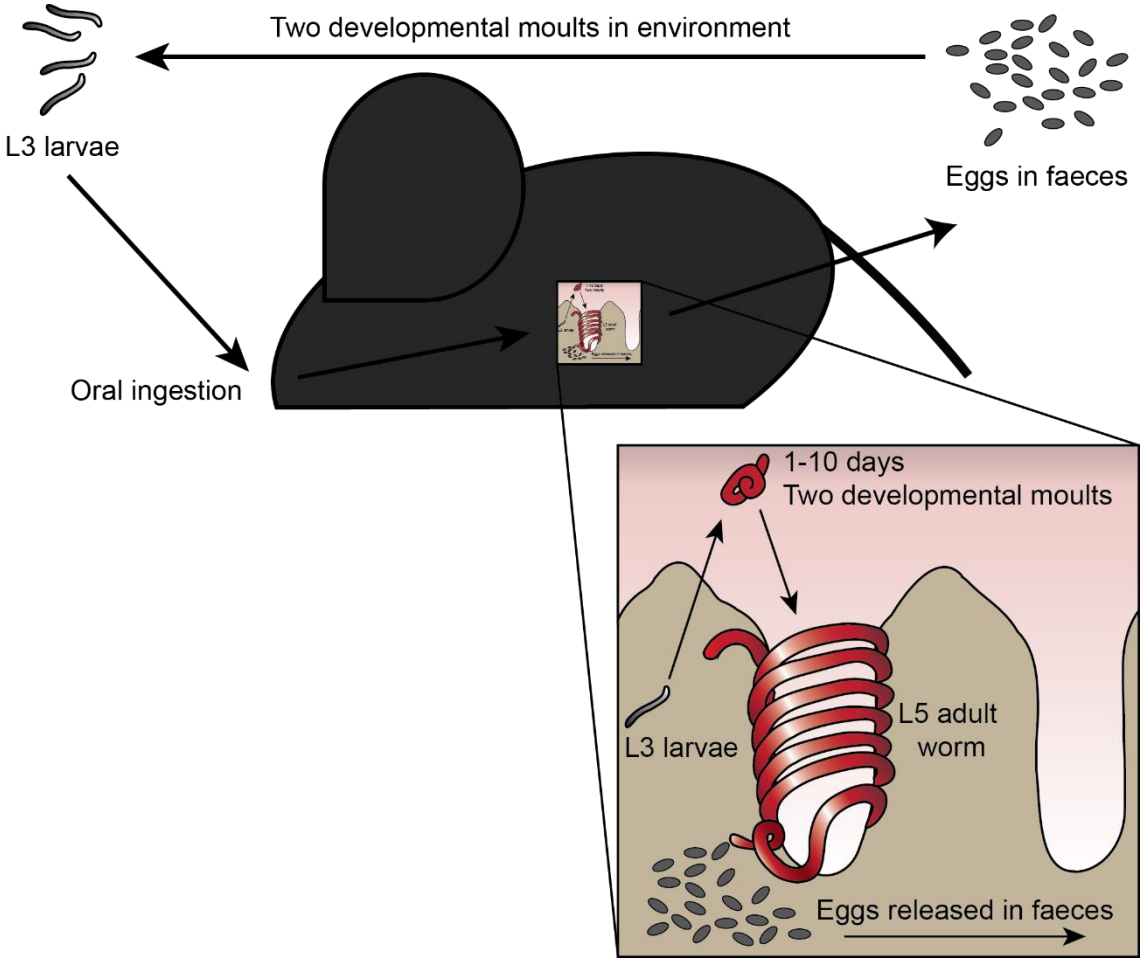


Figure 1.1 *Heligmosomoides polygyrus* lifecycle

Infective L3 *H. polygyrus* larvae are orally ingested by the host and swallowed. The L3 larvae then pass into the small intestine where they penetrate the intestinal mucosa and embed into the *muscularis externa* within 48 hours of infection. Here the larvae undergo two developmental moults (L4 then L5 stage) and re-emerge into the lumen as adult L5 worms after 10 days. Adult worms wrap around the villi to anchor themselves, form mating pairs and produce eggs that are released into the environment in the host's faeces. The eggs hatch in the environment and undergo two developmental moults becoming infective L3 larvae, thus continuing the life cycle.

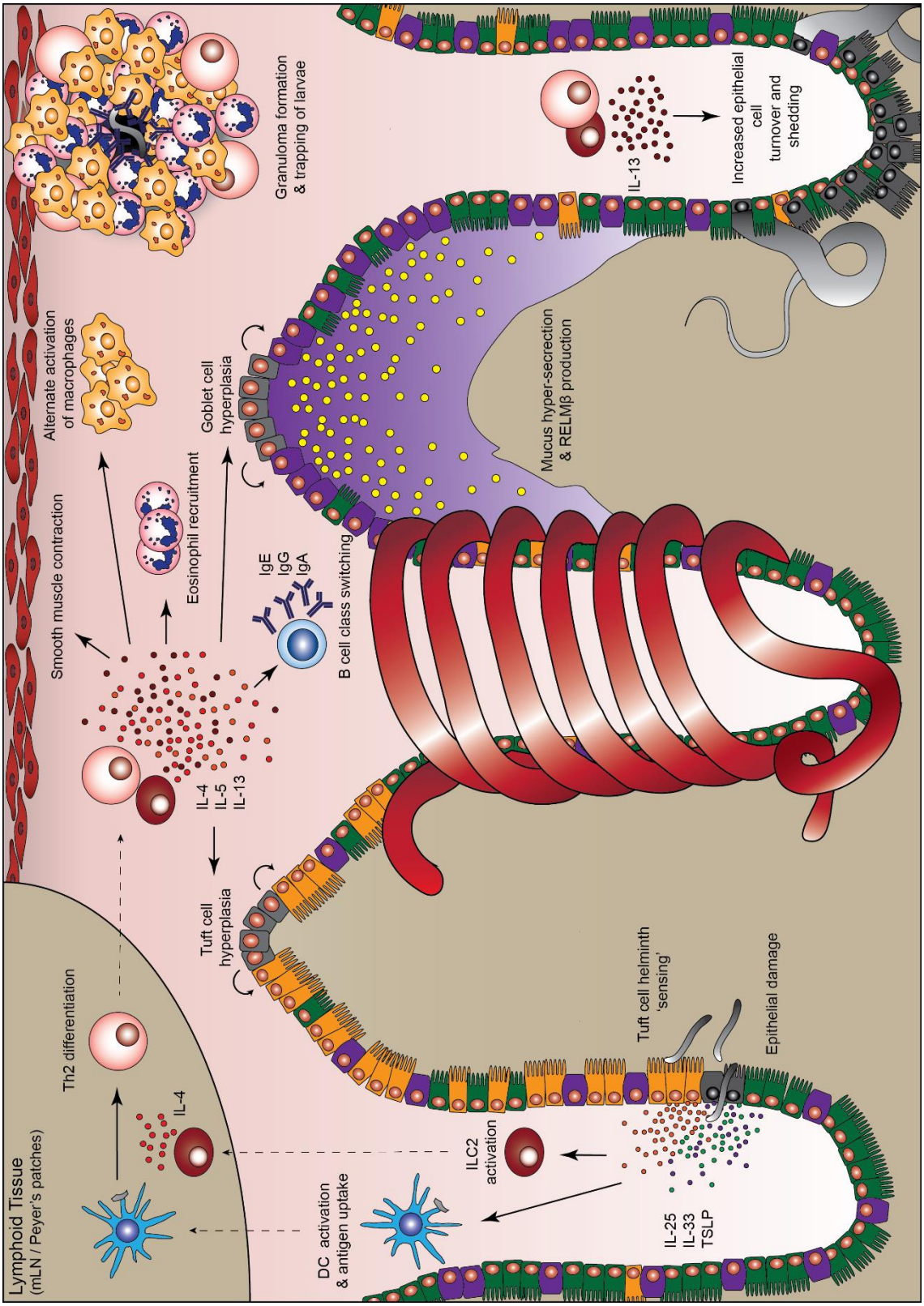

Lymphoid Tissue
(mLN / Peyer's patches)
Th2 differentiation
IL-4
DC activation
& antigen uptake
ILC2
activation
IL-25
IL-33
TSLP
Tuft cell helminth
'sensing'
Epithelial damage
Tuft cell
hyperplasia
IL-4
IL-5
IL-13
Smooth muscle contraction
Alternate activation
of macrophages
Eosinophil recruitment
IgE
IgG
IgA
B cell class switching
Goblet cell
hyperplasia
Granuloma formation
& trapping of larvae
Mucus hyper-secretion
& RELMβ production
IL-13
Increased epithelial
cell
turnover and
shedding

Figure 1.2 Type 2 immune response to intestinal helminth infection

Helminth-induced damage of epithelial cells and helminth 'sensing' by tuft cells induces the secretion of alarmins (TSLP, IL-33 & IL-25). These alarmins promote DC and ILC2 activation as well as antigen uptake. In lymphoid tissues, DC antigen presentation to $CD4^+$ T cells, in the presence of IL-4, promotes Th2 cell differentiation and migration back to the site of infection. Activated ILC2s and Th2 cells release the type-2 cytokines IL-4, IL-5 and IL-13 in the small intestine. These type-2 cytokines activate a variety of stromal and immune cells; goblet cell hyperplasia, increased mucus production, production of Relmβ, Tuft cell hyperplasia, class switching of B cells and production antibodies (IgA, IgE & IgG), alternate activation of macrophages, recruitment of eosinophils, smooth muscle hypercontractility and increased epithelial cell turnover. Activation of stromal cells induce the physical removal of intestinal helminths by the 'weep and sweep' response. The activated and recruited immune cells form granulomas around embedded larvae to promote their killing at the cost of local tissue damage.

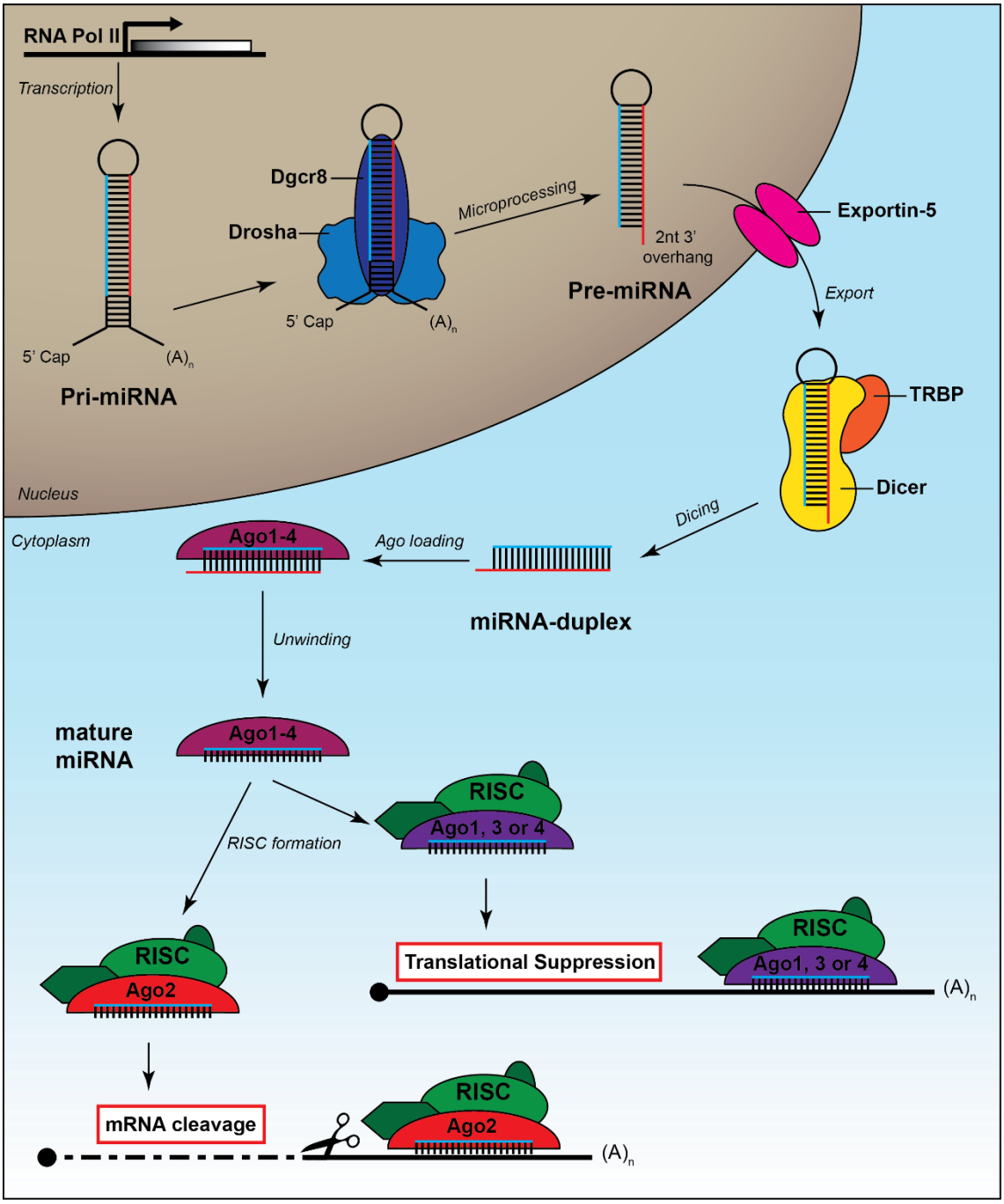

RNA Pol II
Transcription
5' Cap
(A)n
Pri-miRNA
Dgcr8
Drosha
5' Cap
(A)n
Microprocessing
2nt 3' overhang
Pre-miRNA
Exportin-5
Export
TRBP
Dicer
Dicing
Nucleus
Cytoplasm
Ago1-4
Ago loading
miRNA-duplex
Unwinding
mature miRNA
Ago1-4
RISC
Ago1, 3 or 4
RISC formation
RISC
Ago2
Translational Suppression
RISC
Ago1, 3 or 4
(A)n
mRNA cleavage
RISC
Ago2
(A)n

Figure 1.3 Canonical microRNA biogenesis and function

Pri-miRNAs are transcribed by RNA Pol. II from the relevant gene in the nucleus. DiGeorge syndrome critical region gene 8 (Dgcr8) binds the pri-miRNA and recruits the RNase enzyme Drosha. Drosha cleaves the elongated 5' and 3' tails to form pre-miRNA with a 3' overhang of 2nt. The pre-miRNA is then transported out of the nucleus via Exportin-5. In the cytoplasm, TRBP recognises and binds the pre-miRNA structure in complex with the RNase enzyme Dicer. Dicer cleaves the hairpin loop of the pre-miRNA to leave a miRNA-duplex. The miRNA duplex is recognised and bound by one of four Ago proteins and the complementary miRNA strand dissociates to leave a single-stranded, mature miRNA. The miRNA-Ago complex is then loading into the RISC. The miRNA guides the RISC complex to the 3' UTR of the target mRNA, complementary to the miRNA seed sequence. The RISC then binds to the target mRNA and induces 1) translational repression of the target mRNA if Ago protein 1, 3 or 4 are present in the RISC or 2) degradation of the target when Ago2 is present.

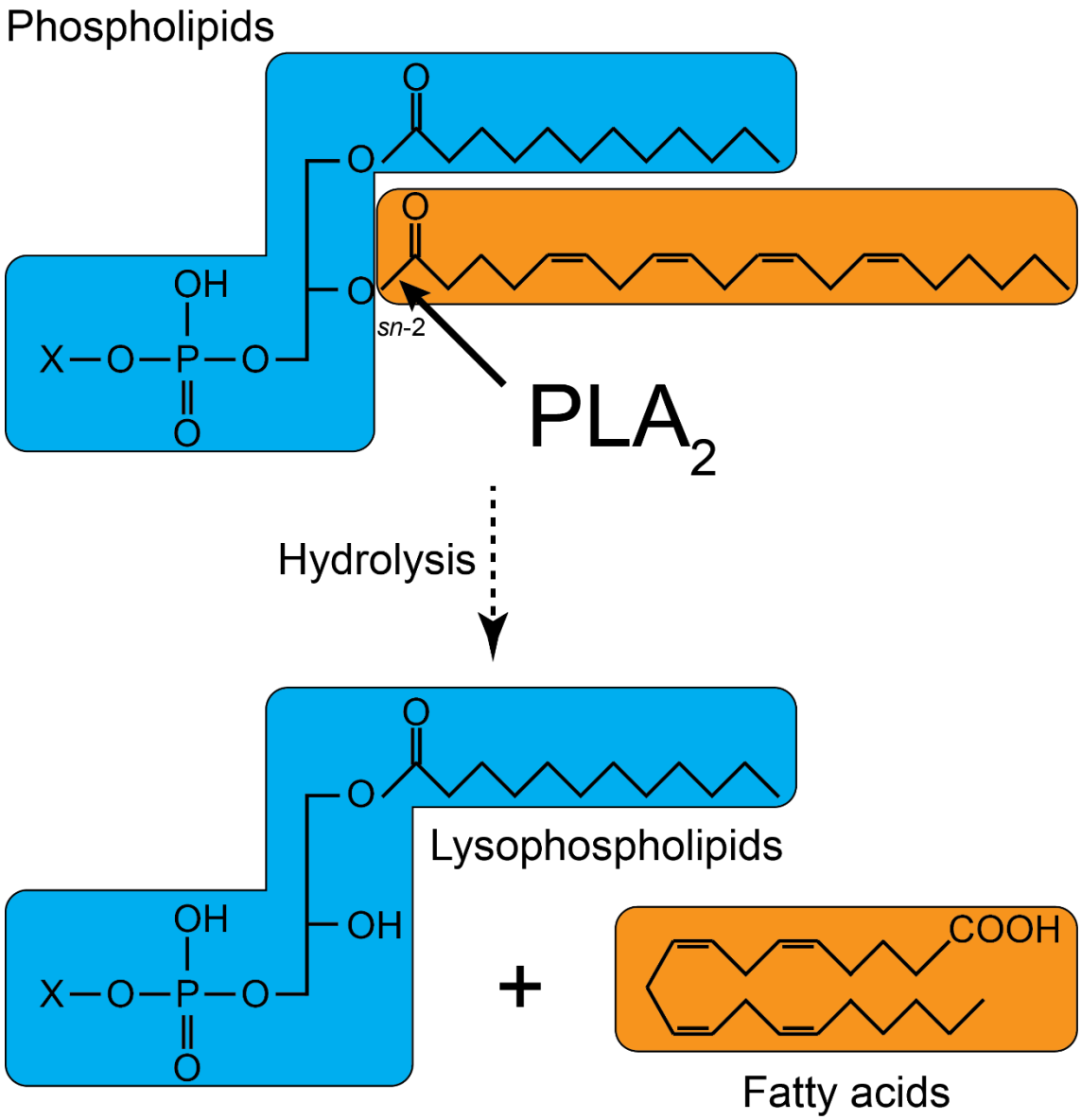


Figure 1.4 Phospholipase A_2 reaction

PLA_2 enzymes hydrolyse phospholipids at the *sn*-2 position to yield lysophospholipids and fatty acids. 'X' determines the phospholipid headgroup.

Chapter 2. Materials & Methods

2.1 Animals

All mice used in this study were maintained under specific pathogen-free conditions at both the Mill Hill Laboratory, The Francis Crick Institute (London, UK) and The Francis Crick Institute, 1 Midland Road (London, UK). C57BL/6, *Pla2g1b*$^{-/-}$ (Richmond et al., 2001), 4get (Mohrs et al., 2001), *Dicer*$^{fl/fl}$ (Murchison et al., 2005), *R26*^{CreERT2} (Ventura et al., 2007), *R26R*^{yfp} (Srinivas et al., 2001) *Rag*$^{-/-}\gamma c^{-/-}$ and *Rag2*$^{-/-}$ mice were bred and maintained at The Francis Crick Institute. *Pla2g1b*$^{-/-}$ *Il4*^{gfp} mice were generated by crossing *Pla2g1b*$^{-/-}$ and 4get mice at The Francis Crick Institute. *R26*$^{CreERT2/yfp}$*Dicer*$^{fl/fl}$ mice were generated by crossing *Dicer*$^{fl/fl}$, *R26*^{CreERT2} and *R26*^{yfp} mice at The Francis Crick Institute. All mice used were male and between 6-12 weeks old at the start of the experiment. Animal experiments were performed according to institutional guidelines and following UK Home Office regulations (project license 70/8809) and were approved by The Francis Crick Institute Ethical Review Panel. Mouse strains were genotyped by PCR analysis of tail or ear DNA using Transnetyx® Automated Genotyping.

2.2 Intestinal helminth infections

2.2.1 *Heligmosomoides polygyrus*

The *H. polygyrus* lifecycle was maintained at The Francis Crick Institute. C57BL/6 lifecycle mice were infected with 200 L3 infective *H. polygyrus* larvae. Faeces and cecal content from infected mice was harvested 14-28 days-post infection and spread onto damp filter paper (Whatmann) with 4-6 pieces of washed, activated charcoal (Sigma). The filter paper was then placed onto a 35 mm petri dish lid inside a 100 mm petri dish with 15 mL of MilliQ Water. The petri dishes were kept at room temperature, protected from light. L3 larvae were harvested from 14 days-post culture.

For experiments, mice were infected with 200 L3 infective *H. polygyrus* larvae (oral gavage). Mice were drug cured (Rx) with the anthelminthic drug Pyrantel Embonate

(2.5 mg/dose, Pfizer) (oral gavage) on days 14 and 15. Mice were secondary (2^o) challenge infected on day 35 or day 56, as indicated, with 200 L3 infective *H. polygyrus* larvae (oral gavage). *H. polygyrus* worms were counted in the wall of the intestine at day 5 post infection and luminal worms were counted in the small intestine 14 days-post 1^o or 2^o infection using a stereoscopic microscope (SMZ-2B, Nikon).

2.2.2 *Nippostrongylus brasiliensis*

The *N.* brasiliensis lifecycle was maintained by Helena Helmby at the London School of Hygiene & Tropical Medicine. Infective L3 larvae were kindly gifted for use at The Francis Crick Institute.

For experiments, mice were infected with 350 L3 infective *N. brasiliensis larvae* (*s.c.*) on day 0. Luminal *N. brasiliensis* worms were counted in the small intestine 6, 8 and 10 days-post infection using a stereoscopic microscope (SMZ-2B, Nikon).

2.2.3 *Trichuris muris*

The *T. muris* lifecycle, originally provided by Prof. Richard Grencis (University of Manchester), was maintained at The Francis Crick Institute. *Rag2*$^{-/-}$ mice were infected with 200 embryonated *T. muris* eggs. The cecum from infected mice were harvested from day 21-35 post infection and the adult worms removed, washed in PBS and incubated at 37^{o}C for 24 hours in RPMI (Gibco), supplemented with400U/ml Penicillin (Gibco) and 400µg/ml Streptomycin (Gibco). Worms were then removed and the remaining media was harvested. The media was centrifuged and the supernatant discarded to leave the eggs. The eggs were washed twice in MilliQ water, passed through a 100 micron filter (Corning) and incubated in 150 mL in a tissue culture flask for 2-3 months protected from light, at room temperature to allow for embryonation.

Mice were infected with 200 embryonated *T. muris* eggs (oral gavage) on day 0. *T. muris* worms were counted in the cecum 35 days-post infection using a stereoscopic microscope (SMZ-2B, Nikon).

2.3 Tamoxifen treatment

Mice were treated with tamoxifen (1mg *i.p.* plus 1mg oral gavage, Sigma) or corn oil (vehicle, Sigma) for 5 consecutive days. Mice were culled three days after the final treatment or infected with *H. polygyrus* and culled 14 days-post infection.

2.4 Antibiotic treatment

The antibiotics Gentamicin sulfate salt (1 mg/mL, Sigma), Metronidazole (1 mg/mL, Sigma), Cefloxin sodium salt (1 mg/mL, Santa Cruz Biotechnology) and Vancomycin hydrochloride (1 mg/mL, Sigma) were administered in the drinking water. Treatment started 7 days prior to 1º *H. polygyrus* infection (Day -7) and maintained throughout the duration of the experiment.

2.5 Antibody treatment

Anti-IL-4 antibody (0.5 mg/dose, BioXcell) was administered *i.p.* on days 13, 15, 17, 19 and 21 after 1º *H.* polygyrus infection. Mice were drug cured (Rx) with the anthelminthic drug Pyrantel Embonate (2.5 mg/dose, Pfizer) *(p.o.*) on days 14 and 15.

2.6 miRNA inhibitor treatment

In vivo miRCURY LNA™ microRNA Inhibitors (Exiqon) were designed and manufactured for inhibiting mmu-miR-99a-5p, mmu-miR-148a-3p and mmu-miR-155-5p. A negative control *in* vivo miRCURY LNA™ microRNA Inhibitor (Exiqon) was also used.

miRNA inhibitors were administered during 2º challenge *H. polygyrus* infection (section **2.2.1**). Mice were given a 1º *H. polygyrus* infection on day 0 and drug cured

on days 14 and 15. Mice were 2^{o} challenge infected with *H. polygyrus* on day 35. miRNA inhibitors were given prior to and during 2^{o} challenge infection on days 33, 35, 37 and 40 (0.125 mg per dose, *s.c.*). Mice were culled on day 49, 14 days-post 2^{o} infection.

2.7 PLA_2g1B treatment

For PLA_2g1B treatment, infective L3 *H. polygyrus* larvae were exsheathed as previously described (Sommerville and Bailey, 1973). 0.85% w/v NaCl (in distilled water) was bubbled with 40% CO_2 in Nitrogen for 5 minutes, the tube was then sealed and incubated in a 37^{o}C waterbath for a further 5 minutes. The pH was adjusted to pH2 using HCl and 5 mL was added to 30000-50000 L3 larvae before bubbling with 40% CO_2 in Nitrogen for 30 seconds. The tube was then sealed and incubated in a 37^{o}C waterbath for a further 30 minutes. Exsheathed L3 larvae were washed and counted before treatment with PLA_2g1B. 8000 exsheathed L3 *H. polygyrus* larvae were treated with recombinant mouse PLA_2g1B (Elabscience) with or without manoalide (200 ng/µL final concentration, Santa Cruz Biotechnology), manoalide alone, or heat inactivated recombinant mouse PLA_2g1B in 1mL EnzChek® PLA_2 reaction buffer (Invitrogen) at room temperature for 24 hours. Recombinant mouse PLA_2g1B was heat inactivated by heating at 100^{o}C for 4 hours. Following treatment, larvae were washed with MilliQ water prior to use in ATP assay (detailed below) or infection. Adult L5 *H. polygyrus* worms were isolated from C57BL/6 following primary infection between days 14 and 28 using a modified Baermann apparatus (Johnston et al., 2015). Adult L5 *H. polygyrus* worms were treated with recombinant mouse PLA_2g1B (Elabscience) or heat inactivated recombinant mouse PLA_2g1B in EnzChek® PLA_2 reaction buffer (Invitrogen) at room temperature for 24 hours.

2.8 ATP assay

The ATP of infective L3 *H. polygyrus* larvae, L4 *H. polygyrus* larvae (removed from intestinal wall at day 7 post infection) or adult L5 *H. polygyrus* worms was measured using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega). *H. polygyrus* adult worms, two L4 larvae or 100 L3 larvae were homogenised using a motorised pestle in 110 µL of PBS and 110 µL of CellTiter-Glo® Reagent. The homogenate was incubated for 10 minutes at room temperature before centrifugation at 1000g for 3 minutes. 200 µL of the supernatant was transferred to a 96 well opaque-walled plate and incubated for 10 minutes at room temperature before recording luminescence. An ATP standard curve was generated by using recombinant ATP (Promega) as detailed in the CellTiter-Glo® Luminescent Cell Viability Assay instructions.

2.9 RNA extraction and analysis

2.9.1 RNA extraction from cells and tissue samples

Tissues were harvested and stored in RNAlater (Sigma) for 24hrs at 4^{o}C before storage at -80^{o}C. For RNA extraction, RNAlater was removed and tissue was homogenised in Qiazol (Qiagen) using the Precellys homogeniser (Bertin Instruments). Cells were lysed and stored in Qiazol (Qiagen) at -80^{o}C. RNA was extracted using the RNeasy Kit (Qiagen), following the manufacturer's instructions. RNA concentration was measured using a ND-1000 Spectrophotometer (NanoDrop Technologies) or Qubit 2.0 Fluorometer (Invitrogen).

2.9.2 mRNA sequencing and analysis

2.9.2.1 Chapter 3 mRNA sequencing and analysis

RNA integrity was confirmed using Agilent's 2100 Bioanalyser. Total RNA libraries were created using the Encore® Complete RNA-Seq Library Systems kit (NuGEN) or TruSeq® RNA Sample Preparation v2 kit (Illumina), following manufacturer's instructions. Total RNA libraries were sequenced using the Illumina® HiSeq 2500.

The raw Illumina reads were analysed as follows. First, the data quality was analysed using FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc). Then the low quality bases were trimmed using Trimmomatic. The read pairs which passed the trimming quality filters were then aligned to mm10 (Ensembl version 75) using Tophat2. Counts were determined using htseq_count. Normalisation and statistical analysis was performed using edgeR. Differential gene analysis was calculated from naïve control group. Statistically significant genes with FDR < 0.05 were reported.

2.9.2.2 Chapter 4 mRNA sequencing and analysis

Biological replicate libraries were prepared using the KAPA polyA stranded mRNA Library Prep Kit and sequenced on Illumina HiSeq 2500 platform, generating ~30 million 75bp single-end reads per sample. The RSEM package (version 1.2.31) (Li and Dewey, 2011) in conjunction with the STAR alignment algorithm (version 2.5.2a) (Dobin et al., 2013) was used for the mapping and subsequent gene-level counting of the sequenced reads with respect to Ensembl mouse GRCm.38.86 version genes. Normalisation of raw count data and differential expression analysis was performed with the DESeq2 package (version 1.10.1) (Love et al., 2014) within the R programming environment (version 3.2.3) (www.r-project.org/). Differentially expressed genes were defined as those showing statistically significant differences (p<0.05).

2.9.3 microRNA sequencing and analysis

RNA integrity was confirmed using Agilent's 2100 Bioanalyser. miRNA libraries were created using the NEBNext Multiplex Small RNA Library Prep Set for Illumina (New England BioLabs), following manufacturer's instructions. miRNA libraries were sequenced using the Illumina® MiSeq. The raw Illumina reads were analysed as follows. First, the data quality was analysed using FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc). Then the low quality bases were trimmed using Trimmomatic. The read pairs which passed the trimming quality filters were then aligned to mirBase (release21) using Novoalign v3.02.12

(http://www.novocraft.com/support/download/). Counts were determined using the following R script.

```
aggregate_mmu_counts <- function (cfile)

{library("plyr")

mmu <- read.table("/Users/nnikolo/Documents/mmu.txt")

colnames(mmu) <- c("mmu","counts")

sample_counts <- read.table(cfile)

colnames(sample_counts) <- c("counts", "mmu")

sample_all_counts <- rbind(mmu, sample_counts)

sample_sum_counts <-
ddply(sample_all_counts,~mmu,summarise,total_counts=sum(counts))

setwd(dirname(cfile))

write.table(sample_sum_counts, file="agg.counts", row.names = FALSE,
col.names = FALSE, sep = "\t", quote = FALSE)

sum(sample_sum_counts$total_counts)}
```

Normalisation and statistical analysis was performed using edgeR script. Differential gene analysis was calculated from naïve control group. Statistically significant miRNAs with FDR < 0.05 were reported.

2.9.4 IPA® analysis

Analysed RNA sequencing data sets were uploaded into Ingenuity Pathway Analysis® (IPA®, Qiagen) where fold change filters, pathway analysis, upstream regulator analysis and miRNA target filter analysis algorithms were applied.

For pathway analysis and upstream regulator analysis algorithms, uploaded expression data was subjected to ‘Core Analysis’. Following Core Analysis, ‘Disease and Bio Functions’ and ‘Upstream Analysis’ algorithms were applied and the predicted activation score extracted and reported.

For miRNA target filter analysis, miRNA- and corresponding mRNA expression datasets were subjected to the ‘microRNA Target Filter’ analysis algorithm with a

minimum confidence level of Moderate (predicted). The results were filtered on miRNA-mRNA expression pairing, specifically 'miRNA up, mRNA down' and 'miRNA down, miRNA up'. Resulting mRNA targets for the relevant miRNA were then extracted and reported.

2.9.5 Ratios of ratios analysis

Ratio of ratios plots were generated from plotting the fold change of genes in *H.p.* 2º relative to *H.p.* 1º (2-fold filter, $p<0.05$) (y axis) against the fold-change of each gene in both *H.p.* 2º and *H.p.* 1º, relative to naïve (2-fold filter, $p<0.05$) (x axis).

2.9.6 mRNA Quantitative real-time polymerase chain reaction

RNA was extracted and purified from tissue or cells as described above. Reverse transcription was performed with 0.1-1 µg RNA using Quantitect RT Kit (Qiagen) following manufacturer's instructions to create cDNA. cDNA was used for quantitative real-time PCR analysis using Power SYBR® Green PCR Master Mix (Applied Biosystems) and quantified on the 7900HT (Applied Biosystems) or QuantStudio5 (Applied Biosystems). The PCR conditions were as follows: 95°C for 15min, 40 cycles of 94°C for 30s, 55°C for 30s, 72°C for 30s followed by a melting step of 95°C for 15s, 60°C for 15s, 95°C for 15s. Where appropriate, relative gene expression was determined via normalisation to the housekeeping gene *Hprt* and the relevant control group (see Figure legends). Please see section **2.24** for primer sequences.

2.9.7 miRNA Quantitative real-time polymerase chain reaction

RNA was extracted and purified from tissue or cells as described above. Reverse transcription was performed with 0.1-1 µg RNA using miSCRIPT II RT Kit, HiSpec buffer, (Qiagen) following manufacturer's instructions to create cDNA. Generated cDNA was used for quantitative real-time PCR analysis using Power SYBR® Green PCR Master Mix (Applied Biosystems) and quantified on the 7900HT (Applied

Biosystems) or QuantStudio5 (Applied Biosystems). The PCR conditions were as follows: 95°C for 15min, 40 cycles of 94°C for 30s, 55°C for 30s, 70°C for 30s followed by a melting step of 95°C for 15s, 60°C for 15s, 95°C for 15s. All miRNA and snoRNA primers were purchased from Qiagen for use with the miSCRIPT II RT Kit. Where appropriate, relative miRNA expression was determined via normalisation to the housekeeping snoRNA RNU6B and the relevant control group (see Figure legends).

2.10 Histopathology

2.10.1 Histology staining

Small intestinal tissue was removed and fixed in 4% formaldehyde for 24 hours then washed in 70% ethanol. The tissues were embedded in paraffin, and sectioned. Sections were stained with haematoxylin and eosin stain or Alcian blue/ periodic acid-Schiff stain. Stained slides were scanned with a VS120-SL slide scanner (Olympus, Tokyo, Japan) and images were captured with the OlyVIA image viewer (Olympus).

2.10.2 *In situ hybridisation*

Pla2g1b staining: *Pla2g1b* RNAscope® probes were designed by Advanced Cell Diagnostics and *in situ* hybridisation was performed with RNAscope® 2.5 Reagent Kit Brown (Advanced Cell Diagnostics), following the manufacturer's instructions. The final *Pla2g1b* signal was detected chromogenically using DAB and the sections were counterstained with Haematoxylin Stained. Stained slides were scanned with a VS120-SL slide scanner (Olympus, Tokyo, Japan) and images were captured with the OlyVIA image viewer (Olympus).

2.11 Small intestine homogenate preparation

1cm of duodenal tissue was removed and homogenised in 300 µL of cOmplete protease inhibitor (Roche), using the Precellys homogeniser (Bertin Instruments), before centrifugation. The supernatant was removed and the protein concentration calculated using the ND-1000 Spectrophotometer (NanoDrop Technologies). The small intestinal homogenate was stored at -80^{o}C.

2.12 PLA_2 activity assay

PLA_2 activity was determined using EnzChek® Phospholipase A_2 Assay Kit (Invitrogen), following manufactures instructions. 25 µL of the small intestinal homogenate (see section **2.11**) was transferred to a ½ well 96 well plate with 25 µL of the substrate-liposome mix then incubated at room temperature in the dark for 10 minutes. Fluorescence emission was measured at 515nm and reported after blank reduction.

2.13 Preparation of single cell suspensions

2.13.1 Isolation of cells from Spleen, mesenteric lymph nodes and thymus

The spleen, mLNs and thymus were made into single-cell suspensions by gently mashing through a 40 micron filter (Thermo-Scientific), and the red blood cells were lysed from the spleen single cell suspension with ACK lysis buffer (Gibco). Cells were then resuspended in cIMDM (complete Iscove's Modified Dulbecco's Medium (cIMDM) containing 1% fetal bovine serum (FBS), 1mM EDTA, 100U/ml Penicillin (Gibco) and 100µg/ml Streptomycin (Gibco), 8mM L-glutamine (Gibco) and 0.05mM 2-mercaptoethanol (Gibco)). Single cell suspensions were used for *ex* vivo restimulations and flow cytometry analysis.

2.13.2 Isolation of intestinal epithelial cells

For the isolation of small intestinal epithelial cells: adipose tissue was removed from the small intestine before being dissected longitudinally to remove faecal contents and cut into 2cm segments. The epithelial layer was then dissociated by incubating the intestine segments in PBS containing 10% FBS, 15 mm HEPES, 5 mm EDTA (Life Technologies) and 1 mm dithiothreitol (Sigma) for 30 minutes at 37 °C. The remaining intestinal tissue was removed using a wide mesh sieve and epithelial layer was retained. Cells were layered onto 20% isotonic Percoll (GE Healthcare) to remove debris. Cells were then resuspended in complete Iscove's Modified Dulbecco's Medium (cIMDM) containing 1% fetal bovine serum (FBS), 1mM EDTA, 100U/ml Penicillin (Gibco) and 100µg/ml Streptomycin (Gibco), 8mM L-glutamine (Gibco) and 0.05mM 2-mercaptoethanol (Gibco)) and prepared for cell sorting.

2.13.3 Cell counting

Aliquots of single cell suspensions were diluted appropriately in cIMDM. Samples were then mixed at a 1:1 ratio in Trypan blue (Sigma). Live cells were counted using a cell counting haemocytometer (Hawksley) and a LED inverted light microscope (Leica).

2.14 Fluorescence activated cell sorting (FACS) and flow cytometry

Cell sorting was performed using a MoFlo XDP cell sorter (Beckman Coulter). Cell suspensions were stained for 25 minutes with antibodies in PBS with 1% FCS. To prepare for sorting, stained cells were diluted in phenol-red free IMDM (Gibco) (with 1% FCS, 2mM EDTA (Invitrogen), 100 U/mL Penicillin and 100 µg/mL Streptomycin (Gibco), 8 mM L-glutamine (Gibco), and 0.05 mM 2-mercaptoethanol (Gibco)). For flow cytometry analysis, cells were analysed using a BD LSRFortessa™ X-20 (BD Biosciences) or BD LSRII (BD Biosciences) and data were analysed using FlowJo

software (Version 10, Treestar Inc). Cells were sometimes fixed in 2–4% paraformaldehyde for Flow cytometry analysis.

For cell sorting, viability of the cells was determined using Propidium Iodide (Sigma); for analysis, viability of the cells was determined using the LIVE/DEAD Fixable Blue kit (Life Technologies). All staining was performed in the presence of FcR Blocking Reagent (Miltenyi Biotec).

Intracellular cytokine staining (ICS) was performed following 6 hours of re-stimulation with 50ng/mL phorbol 12-myristate 13-acetate (PMA, Promega) and 1 µg/mL ionomycin (Sigma) and BD Golgi Stop and BD Golgi Plug (diluted 1:1000, BD Biosciences). Following surface stain, cells were incubated with Fixation/Permeabilization buffer (eBioscience) for 25 minutes followed by 25 minutes in Permeabilization buffer (eBioscience), and incubation with antibodies in Permeabilization buffer for a further 30 minutes.

ILCs were analysed using the following strategy: Live, lymphocytes, $CD45^+$, $Lineage^-$ (CD3, CD4, CD8, CD19, CD11c, CD11b, NK1.1, TCRβ, TCRγδ, Gr-1, CD49b, Ter119), $Thy1.2^+$, $KLRG1^+$, and $Sca1^+$.

Tregs were analysed using the following gating strategy: Live, lymphocytes, $CD4^+$, $TCR\beta^+$, $CD25^+$ and $Foxp3^+$.

Epithelial cells were sorted using the following gating strategy: Live, $CD45^-$ and $EpCam^+$.

2.15 *In vitro* cell culture and stimulation assays

2.15.1 Bone marrow-derived macrophages culture and stimulation

Bone marrow was isolated from the femur and tibia of mice and the red blood cells were lysed with ACK lysis buffer (Gibco). The remaining cells were then cultured in DMEM (Gibco) (with 20% L929 cell media (in-house preparation), 10% FCS (Invitrogen), 1% L-Glutamine (Gibco), 100 U/mL Penicillin and 100 µg/mL Streptomycin (Gibco), 10 mM HEPES (Lonza) and 0.05mM 2-mercaptoethanol (Gibco)) in 10mls at a density of $5x10^5$ cells/ml at 37°C. After 7 days of culture non-adhesive cells were removed before removing adherent BMDMs using 2.5mM EDTA

(Invitrogen) in PBS (Gibco) with 5% FCS (Invitrogen). Adherent BMDMS were washed and resuspended in DMEM (with 1% FCS (Invitrogen), 1% L-Glutamine (Gibco), 100 U/mL Penicillin and 100 µg/mL Streptomycin (Gibco), 10 µM HEPES (Lonza) and 0.05mM 2-mercaptoethanol (Gibco)). Adherent BMDMs were then plated at a density of $2x10^6$ cells/ml for 24 hours. The adherent BMDMs were then stimulated for 24 hours with either 20ng/ml IL-4 (R&D) and 20ng/ml IL-13 (R&D) before RNA extraction.

2.15.2 *Ex vivo* stimulations

mLNs were harvested and processed into a single cell suspension (see section **2.12.1**). Cells were plated at $2x10^5$ cells per 200µl cIMDM with 10µg/ml of *H. polygyrus* antigen extract (HEX) at 37°C. Supernatant was harvested after 4 days. Cytokines were detected in the supernatant using ELISAs.

For HEX preparation, mixed sex adult L5 *H. polygyrus* worms were removed from the small intestine of infected mice and homogenised using a ground-glass homogeniser (Jencons H103/32/324) in 1ml of PBS, in the absence of protease inhibitors. The homogenate was centrifuged at 10000 g for 20 minutes. The soluble supernatant fraction was passed through a 0.2µm filter (Millipore) prior to protein concentration determination using the Pierce BCA protein assay (ThermoFisher). Hex was stored at -80°C until use.

2.15.3 Intestinal organoid culture – in collaboration with Amina Metidji

Mouse organoids were established and maintained at 37°C as three-dimensional spheroid culture in Matrigel (R&D system) from isolated crypts collected from the duodenum. The basic culture medium (ENR) contained advanced DMEM/F12 supplemented with penicillin/streptomycin, 10 mM HEPES, 2mM Glutamax, B27 (all from Life Technologies) and 1 mM N-acetylcysteine (Sigma) supplemented with murine recombinant EGF (life technologies), R-spondin1-CM (Trevigen) (10% final volume) and Noggin-CM (kindly provided by Dr. Hans Clevers, Hubrecht Institute, Utrecht, The Netherlands) (20% v/v). Wnt3a-CM was used at 50% (v/v) for 7 days at

the beginning of the culture, then withdrawn. Organoids were stimulated with 20 ng/mL IL-4 (R&D) and 20ng/mL IL-13 (R&D) or small intestinal homogenate (see section **2.11**) for 48 hours. RNA was extracted and qRT-PCR performed as described in section **2.9.6**.

2.16 ELISAs and EIAs

2.16.1 Cytokines

IFNγ, IL-5 and IL-13 were measured using DuoSet ELISA kits, according to the manufacturer's instructions (R&D).

2.16.2 IgE

Total IgE ELISA was performed by coating with Purified Rat Anti-Mouse IgE (R35-72, BD Pharmingen) at 2 µg/mL overnight, followed by overnight incubation with serum and standard (Purified Mouse IgE, k isotype Standard, BD Pharmingen), and detection with Biotin Rat Anti-Mouse IgE at 1 µg/mL (R35-118, BD Pharmingen), Streptavidin HRP at 1:000 (BD Pharmingen) and ABTS One Component HRP Microwell Substrate (SurModics).

2.16.3 IgG1

H. *polygyrus*-specific IgG1 was detected by coating plates with 5 µg/mL *H*. *polygyrus* antigen extract (HEX) overnight, followed by overnight incubation with serially diluted serum and detection with Biotin Rat Anti-Mouse IgG1 (Invitrogen), Streptavidin HRP at 1:1000 (BD Pharmingen) and ABTS One Component HRP Microwell Substrate (SurModics).

2.16.4 Eicosanoids

Cysteinyl leukotrienes and prostaglandin E_2 were measured in small intestinal homogenate (see section **2.11**) using ELISA kits, according to manufacturer's instructions (Enzo), and normalised to total protein content.

2.17 Serum chemistry analysis

Whole blood was collected from mice and the serum separated after clotting. The serum was sent to the MRC Harwell Institute (UK) for metabolite analysis using a Beckman Coulter AU680 clinical chemistry analyser.

2.18 Lysophosphatidylcholine Assay

Lysophosphatidylcholine (LPC) was measured in the serum and small intestine homogenate (see section **2.11**) using the AZWELL LPC Assay Kit, according to the manufacturer's instructions (Cosmo Bio), and normalised to total protein content.

2.19 Lipid extraction and analysis – in collaboration with Mariana Silva dos Santos and James MacRae

Lipids were extracted from *H. polygyrus* L3 larvae using a method adapted from a previous publication (Meyer et al., 1966). Following PLA_2g1B treatment ~8000 *H. polygyrus* larvae were washed three times with MilliQ water, resuspended in 2 mL methanol and heated in a sealed tube under nitrogen at 55^{o}C for 20 min. After cooling, 4 mL chloroform was added and the sample was agitated with a magnetic stirrer for 3 hrs. The organic phase was removed and the residue ground (using a glass homogeniser) and extracted with 2 mL chloroform/methanol (2:1, v/v) for 2 hrs.

For LC-MS, lipids were dried under nitrogen and redissolved in 100 µL methanol/chloroform (1:1 v/v) and diluted 1:2 with solvent A (hexane:isopropanol, 70:30 [v: v], 0.02% [m/v] formic acid, 0.01% [m/v] ammonium hydroxide), centrifuged

at 1,500 rpm for 5 min to remove trace non-lipidic materials prior to transfer to a glass autosampler vial (Agilent). 10 µL was injected onto a BETASIL diol column (5 um x 150 mm x 2.1 mm, with BETASIL diol guard column (10 mm x 2.1 mm), held at 20°C) in an Ultimate 3000 HPLC system coupled to a Thermo Exactive Plus Orbitrap MS for full scan or Q Exactive Orbitrap MS for MS/MS scan. Lipids were eluted at 0.15 ml/min with a binary gradient from 0% to 100% solvent B (isopropanol:methanol, 70:30 [v/v], 0.02% [m/v] formic acid, 0.01% [m/v] ammonium hydroxide): 0–10 min, 0% B; 17–22 min, 50% B; 30–35 min, 100% B; 40–44 min, 0% B, followed by additional 6 min 0% B post-run. MS data were acquired in both polarities using a full scan method. The positive and negative HESI-II spray voltages were 4.5 and 3.5 kV, respectively; the heated capillary temperature was 250°C; the sheath gas pressure was 30 psi; the auxiliary gas setting was 20 psi; and the heated vaporizer temperature was 150°C. Both the sheath gas and the auxiliary gas were nitrogen. The parameters of the full mass scan were as follows: a resolution of 70,000, an auto gain control target under 3×10^6, a maximum isolation time of 200 ms, and an m/*z* range 200–3000. Where possible, to confirm the identification of significant features, samples were re-run in parallel reaction monitoring (PRM) mode, parameters as follows: a resolution of 17,500, an auto gain control target under 2×10^5, a maximum isolation time of 100 ms, an isolation window of m/*z* 0.4 and normalized collision energy were optimized for each feature individually. Data were acquired using Xcalibur 3.0.63 (Thermo Fisher Scientific) and Progenesis (Nonlinear Dynamics) was used for data alignment and peak detection. Data were normalized against the total ion abundance.

Annotations were assigned to accurate masses with a maximum error of 5 ppm using Metlin, LipidMaps, Kegg and HMDB which were searched simultaneously using the CEU Mass Mediator engine (http://ceumass.eps.uspceu.es/mediator/).

2.20 Scanning electron microscopy

H. polygyrus larvae were dehydrated stepwise in ethanol (2 x 5 mins in 70, 90 and 100% ethanol). Larvae were critical point dried from 100% Ethanol in a CPD300 critical point drier (Leica Microsystems UK), mounted on a carbon sticky pad on a

stub, sputter-coated with 5 nm of platinum, and imaged in a Phenom ProX benchtop scanning electron microscope (Phenom-World) with a secondary electron detector.

2.21 Statistical analysis

All statistical analysis for biological data was performed using GraphPad Prism (v6.02). Data was analysed, where appropriate, with either an unpaired one-tailed t test, unpaired two-tailed t test, One-way ANOVA (Tukey's or Dunnett's multiple comparison analysis), Two-way ANOVA (Sidak's or Dunnett's multiple comparison analysis) or Mann-Whitney test. n represents the number of biological replicates. Please see figure legends for statistical tests used and exact value of n. Values are reported as the means ± SEM. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$ and **** = $p<0.0001$.

2.22 Data availability

Description: RNA sequencing data comparing susceptible and resistant mice (**Figure 3.1**) (**RNAseq of duodenal tissue from C57BL/6 mice both susceptible and resistant to Heligmosomoides polygyrus**). The raw and analysed RNA sequencing data files have been deposited in the NCBI Gene Expression Omnibus database under ID code GSE102789.

Description: LC-MS data comparing PLA_2g1B-treated and control-treated *H. polygyrus* L3 larvae (**Entwistle1_archive.zip**). The metabolomics data have been deposited in the EMBL-EBI MetaboLights database under ID code MTBLS523 and PubMed PMID: 23109552.

2.23 Flow cytometry antibodies

Marker	Clone	Conjugate	Manufacturer	[µg/mL]
CD3	145-2C11	APC	BioLegend	1

CD4	RM4-5	BV605, eFluor450,	eBioscience	1
CD4	RM4-5	APC	BioLegend	0.5
CD8	53-6.7	PE-Cy7, APC	BioLegend	0.5-1
CD11b	M1]70	APC	BioLegend	0.2
CD11c	N418	APC	BioLegend	1
CD19	1D3	eFluor450	eBioscience	1
CD19	6D5	APC	BioLegend	0.5
CD25	PC61	APC-Cy7	BioLegend	1
CD25	PC61	APC	eBioscience	1
CD44	IM7	PerCPCy5.5	eBioscience	1
CD45	30-F11	FITC	eBioscience	1
CD49b	DX5	APC	BioLegend	0.5
CD62L	MEL-14	APC	eBioscience	1
CD69	H1.2F3	PE	BioLegend	1
EpCam	G8.8	APC	eBioscience	1
Foxp3	FJK-16S	PE	eBioscience	1
Gr1	RB68C5	APC	BioLegend	0.5
IFNγ	XMG1.2	PE	BD Bioscience	1
IL-4	11B11	PE	eBioscience	1
IL-5	TRFK5	APC	BD Bioscience	1
IL-13	eBio13A	eFluor660	eBioscience	1
IL-17A	17B7	PE-Cy7	eBioscience	1
KLRG1	2F1	PerCP-eFluor710	eBioscience	1
NK1.1	PK136	APC	BioLegend	1
Sca1	E13-161.7	PB	eBioscience	2.5
TCRγδ	GL3	APC	BioLegend	1
TCRαβ	H57-597	APC	eBioscience	1
TCRαβ	H57-597	PerCPCy5.5	BioLegend	1
Ter119	TER-119	APC	BioLegend	1

Thy1.2	53-2.1	PE-Cy7	BioLegend	0.5

2.24 qRT-PCR primers

Gene	Forward primer	Reverse primer
Arg1	GGAAAGCCAATGAAGAGCTG	GCTTCCAACTGCCAGACTGT
Chi3l3	CATGAGCAAGACTTGCGTGAC	GGTCCAAACTTCCATCCTCCA
Gob5	CATCGCCATAGACCACGACG	TTCCAGCTCTCGGGAATCAAA
Hprt	GCCCTTGACTATAATGAGTACTTCAGG	TTCAACTTGCGCTCATCTTAGG
Pla2g1b	CTCGGGCCGTGTGGCAGTTC	TGCCGAGCCAGAGCACGAGT
Retnla	CCCTCCACTGTAACGAAGACTC	CACACCCAGTAGCAGTCATCC
Retnlb	ATGGGTGTCACTGGATGTGCTT	AGCACTGGCAGTGGCAAGTA

Chapter 3. Results 1: Phospholipase A_2 group 1B is an endogenous anthelmintic, essential for immunity to *Heligmosomoides polygyrus*

3.1 Introduction

Intestinal helminth infections are highly prevalent in developing counties, infecting approximately a third of the world's human population, causing significant host morbidity (Bethony et al., 2006, Hotez et al., 2008). With no current vaccine, a limited number of effective chemotherapeutic drugs available and the emergence drug-resistance (Albonico et al., 2003, Taman and Azab, 2014), it is essential to further our understanding of mechanisms of antihelminth immunity to provide new avenues of therapeutic intervention.

Our current understanding of protection against intestinal helminth infections places type 2 immunity at the forefront (Allen and Maizels, 2011, Reynolds et al., 2012, Maizels et al., 2012b). Following infection, infective helminth larvae penetrate through the mucosa, inducing local tissue damage, which is detected by specialised epithelial cells, including the recently identified tuft cell. Activated epithelial cells initiate type 2 immunity through the secretion of the alarmins interleukin (IL)-25, IL-33 and TSLP (Gerbe et al., 2016, von Moltke et al., 2016, Howitt et al., 2016, Kim et al., 2013, Wills-Karp et al., 2012). DC activation, antigen uptake and presentation, alongside ILC2 activation and IL-4 secretion, promote the differentiation of naïve $CD4^+$ T cells into T helper Th2 cells following helminth infection (Le Gros et al., 1990, Hsieh et al., 1992, Swain et al., 1990, Pelly et al., 2016). Th2 cell, and ILC2, production of type 2 cytokines IL-4, IL-13 and IL-5 initiate a suite of immunological and physiological changes to promote helminth killing and expulsion (Urban et al., 1991a, Urban et al., 1991b, Pelly et al., 2016). These responses are IL-4Rα-dependent and include B cell class switching and antibody production (McCoy et al., 2008), alternate activation of macrophages (Anthony et al., 2006), goblet cell hyperplasia and Relmβ production (Herbert et al., 2009). Despite these advances in our knowledge of anti-helminth immunity, the precise mechanism(s) of helminth damage and killing in the tissue remain unclear.

In this chapter, we investigated novel mechanisms of antihelminth immunity, utilising the naturally occurring, strictly enteric, murine intestinal helminth *H. polygyrus*. Using *H. polygyrus* allows us to model both susceptibility and resistance in the same strain of mice, removing genetic differences, as C57BL/6 are susceptible to 1^{o} infection (Reynolds et al., 2012), but after drug-clearance of the 1^{o} infection, they are resistant to 2^{o} challenge infection (Finkelman et al., 1997). To identify novel mechanisms of antihelminth immunity in this model, we utilised RNA sequencing of the small intestinal tissue, from mice both susceptible (Naïve and 1^{o}) and resistant (1^{o} + Rx (drug-cured mice) and 2^{o}) to *H. polygyrus* infection, to identify transcriptional changes at the site of infection.

3.2 Results

3.2.1 RNA sequencing identified *Pla2g1b* to be upregulated in the small intestine of mice resistant to *H. polygyrus* infection

3.2.1.1 RNA sequencing of small intestine reveals distinct gene clusters associated with susceptibility and resistance to *H. polygyrus* infection

We infected C57BL/6 mice with *H. polygyrus* L3 larvae, which are naturally susceptible to a 1^{o} infection (Reynolds et al., 2012). A cohort of mice was sacrificed at day 7 post 1^{o} infection (*H.p.* 1^{o}). The remaining mice were drug cured (Rx) of the 1^{o} infection on days 14 and 15, promoting resistance to subsequent secondary (2^{o}) *H. polygyrus* challenge infection (Finkelman et al., 1997). Some mice were culled on days 42 and 63 after drug-cure (Rx (D42) and Rx (D63), respectively). Other mice were 2^{o} challenge infected on day 35 or day 56 and sacrificed 7 days later (*H.p.* 2^{o} (D42) and *H.p.* 2^{o} (D63), respectively). At each time point, duodenal tissue was dissected and the RNA extracted for RNA sequencing (**Figure 3.1.A**). Resistance to 2^{o} *H.* polygyrus challenge infection correlated with increased immune cell infiltrate in the small intestine, compared to *H.p.* 1^{o} (**Figure 3.1.B**). Following RNA sequencing of the duodenal tissue, we identified significantly more transcriptional activity in *H.p.* 2^{o} (D42) and *H.p.* 2^{o} (D63) than *H.p.* 1^{o} (relative to naïve, p<0.05) (**Figure 3.1.C**). Following application of a 2-foldchange filter, 665 genes were differentially expressed in *H.p.* 2^{o} (D42) compared to 145 genes in *H.p.* 1^{o}, with 116 common genes (relative

to naïve, 2-fold filter, $p<0.05$), again correlating with resistance to infection (**Figure 3.1.D**). To further interrogate the transcriptional profile of susceptible and resistant mice, we utilised a ratio of ratios analysis comparing *H.p.* 1° and *H.p.* 2° (D42) (relative to naïve, 2-fold filter, $p<0.05$). With this analysis, we identified three distinct gene clusters (C1-C3) (**Figure 3.1.E**, **Table 3.1-3.3**). Cluster 1 (C1) identified common and quantitative differences between 1° and 2° infection, including several genes previously described in immunity to *H. polygyrus*, such as Arginase 1 (*Arg1*) and Relmβ (*Retnlb*) (Anthony et al., 2006, Herbert et al., 2009). Cluster 2 (C2) identified qualitative differences between susceptible and resistant mice, highlighting genes upregulated in 2° infection only. Cluster 3 (C3) identified qualitative differences downregulated in 2° infection only. Many of these genes in C2 and C3 have not previously been described in immunity to *H. polygyrus*.

3.2.1.2 Transcriptomic analysis highlights potential novel regulators of immunity to *H. polygyrus*

Utilising Ingenuity Pathway Analysis® (IPA®) software, we identified that the transcriptomic profile of *H.p.* 2° (D42) inferred a greater probability of 'disease and biological functions' pathways being activated, such as 'Immune Cell Trafficking' and 'Immunological Disease', than in *H.p.* 1° (**Figure 3.2.A**). This was indicative of both the increased transcriptional activity (**Figure 3.1.D)** and increased speed and magnitude of the type 2 immune response seen in 2° *H. polygyrus* challenge infection (Pelly et al., 2017, Morimoto et al., 2004). Correlating with the different transcriptomic landscapes of the small intestine in *H.p.* 1° and *H.p.*2° infections, we identified different predicted upstream regulators, molecules predicted to be responsible for changes in the transcriptional landscape. For example, predicted upstream regulators during *H.p.*2° (D42) included the type 2 cytokines IL-4, IL-5 and IL-13, the type 2 cytokine-mediated genes Relmβ (*Retnlb*), tissue repair cytokine TGFβ, the bacterial product LPS and broad inflammatory cytokines CSF2 (GM-CSF), TNFα, IL-6 and IL-1β (**Figure 3.2.B**). The type 2 immunity-associated upstream regulators observed in *H.p.*2° (D42) were not observed in *H.p.* 1°, but were instead replaced with type 1 immunity-associated molecules, including IFNγ and TNFα (**Figure 3.2.B**). These data agree with previous reports citing a slow induction of a type 2 response

upon 1º infection and rapid memory type 2 response in 2º *H. polygyrus* infection (Pelly et al., 2016, Pelly et al., 2017, Morimoto et al., 2004). Several upstream regulators were predicted to be inhibited, with Foxa2 predicted to be inhibited during both *H.p.* 1º and *H.p.* 2º infection (**Figure 3.2.B**). Overall, these analyses contribute to the prevailing model of resistance to *H. polygyrus* infection; resistance is mediated by quantitative differences in the type 2 immune response.

3.2.1.3 Lipid metabolism pathways and *Pla2g1b* expression are upregulated in mice resistant to *H. polygyrus*

To investigate differences between susceptible and resistant mice, we analysed the most differentially activated pathways between *H.p.* 1º and *H.p.* 2º infection. This pathway analysis reflected our previous observations (**Figure 3.1, 3.2**) of increased immune-activated pathways, but also identified increased activation of lipid metabolism pathways in *H.p.* 2º (**Figure 3.3.A**), previously described to be essential in alternate activation of macrophages and antihelminth immunity (Huang et al., 2014). Interestingly, increased activation of lipid metabolism pathways were maintained in resistant mice, with or without 2º challenge infection, until day 63 (48 days post drug treatment) (**Figure 3.3.B**). This observation correlates with long-term resistance to 2º challenge infection following drug-treatment (Urban et al., 1991b). Within Cluster 2 (C2), unique genes expressed in *H.p.* 2º infection (**Figure 3.1.E**, **Table 3.2**), we identified elevated expression of *Pla2g1b*, which encodes the enzyme involved in lipid metabolism, phospholipase A_2 group 1B. *Pla2g1b* expression was not upregulated in *H.p.* 1º compared to naïve, but was significantly upregulated following drug treatment, with or without 2º challenge infection, correlating with increased activation of lipid metabolism pathways and resistance to *H. polygyrus* (**Figure 3.3.C**). PLA_2 enzymatic activity was also significantly increased in the small intestine of resistant mice (**Figure 3.3.D**). Despite *Pla2g1b* being the most abundant PLA_2 isoform expressed in *H.p.* 2º, other isoforms of PLA_2 enzymes were differentially expressed in the small intestine upon infection with *H. polygyrus*, most likely contributing to the overall PLA_2 activity in the small intestine (**Figure 3.3.E**).

3.2.2 *Pla2g1b*-deficiency does not alter the immune compartment

Before testing the role of PLA_2g1B in resistance to *H. polygyrus* infection, we first determined whether *Pla2g1b*$^{-/-}$ mice had any intrinsic immune cell defect prior to infection. T cell development to $CD4^+CD8^+$ stage was unaffected in *Pla2g1b*$^{-/-}$ mice (**Figure 3.4.A**). T cell maturation to single positive T cells and egress into peripheral organs was also unaffected by *Pla2g1b*-deficiency with similar frequencies of naïve- and activated $CD4^+CD8^-$ or $CD4^-CD8^+$ T cells in *Pla2g1b*$^{-/-}$ mice (**Figure 3.4.B-E**). $Foxp3^+$ Tregs, B cells and ILC2s in the mLNs were also unaffected in *Pla2g1b*$^{-/-}$ mice (**Figure 3.4.F-H**).

3.2.3 *Pla2g1b* is essential for resistance against small intestinal helminths

To formally test the role of PLA_2g1B in immunity to *H. polygyrus*, we subjected *Pla2g1b*$^{-/-}$ mice to 2º *H. polygyrus* challenge infection. Unlike WT mice, *Pla2g1b*$^{-/-}$ mice failed to expel 2º challenge infection, instead establishing a patent infection (**Figure 3.5.A-B**). We then set to determine whether PLA_2g1B was required for immunity against other small and large intestinal helminths. *Pla2g1b*$^{-/-}$ mice had a delayed expulsion of the mouse adapted, small intestine-dwelling parasite *N. brasiliensis*, with intestinal worms persisting 8 days-post infection (**Figure 3.5.C**). However, PLA_2g1B was not required for expulsion of the large intestine-dwelling whipworm *T. muris*, with *Pla2g1b*$^{-/-}$ mice expelling all luminal worms 35 days-post infection (**Figure 3.5.D**).

3.2.4 Type 2 immunity is intact in *Pla2g1b*-deficient mice following *H. polygyrus* infection

3.2.4.1 *Pla2g1b*-deficiency does not alter $CD4^+$ T cell response to 2º *H. polygyrus* infection

Following the observation that PLA_2g1B was essential for immunity to *H. polygyrus*, and that type 2 immune responses are required for expulsion, we set out to determine whether *Pla2g1b*-deficiency was required for type 2 memory response. Specifically,

Th2 cell differentiation and type 2 cytokine production essential in orchestrating protective immunity to *H. polygyrus* (Urban et al., 1995, Urban et al., 1991b, Urban et al., 1991a). To monitor differentiation and commitment to the Th2 lineage in *Pla2g1b*$^{-/-}$ mice, we inter crossed *Pla2g1b*$^{-/-}$ mice with *Il4*^{gfp} reporter mice (Mohrs et al., 2001). We also analysed cellular IL-4 protein production by intracellular cytokine staining (ICS). No significant difference in frequency or number of *Il4*^{gfp}-expressing T cells in the mLN or spleen 7 days-post 2^{o} *H. polygyrus* infection was observed between WT or *Pla2g1b*$^{-/-}$ mice (**Figure 3.6.A-B**). Neither was there any significant difference in number or frequency of IL-4^{+}CD44^{hi}CD4^{+} T cells between genotypes (**Figure 3.6.C**). Interestingly, we identified a greater number of IL-4^{+}CD44^{hi}CD4^{+} T cells in the *Pla2g1b*$^{-/-}$ mice (**Figure 3.6.D**), despite previously identifying no significant difference in the frequency or number of *Il4*^{gfp}-expressing T cells. This discrepancy between the ICS and *Il4*^{gfp} reporter data maybe due to the potential differences in comparing protein production and transcriptional activity. The increase in number of IL-4^{+}CD44^{hi}CD4^{+} T cells in the *Pla2g1b*$^{-/-}$ mice is perhaps due to the perturbation of resistance in *Pla2g1b*$^{-/-}$ mice (**Figure 3.5.A-B**), therefore promoting an increased Th2 response in an attempt to clear the infection. *Pla2g1b*-deficency did not alter the frequency of Th1 or Th17 cells following infection, with a similar frequency of IFNγ^{+}- and IL-17A^{+}CD44^{hi}CD4^{+} T cells in *Pla2g1b*$^{-/-}$ mice (**Figure 3.6.C**). However, we saw an increased number of IFNγ^{+}CD44^{hi}CD4^{+} T cells following 2^{o} *H. polygyrus* infection in *Pla2g1b*$^{-/-}$ mice (**Figure 3.6.D**).This may be due to the PMA and ionomycin restimulation required to identify cytokine production by ICS as *H. polygyrus* antigen stimulation induced no significant difference in IFNγ production (**Figure 3.6.E**). PMA and ionomycin restimulation is fundamentally different to antigen-specific stimulation; PMA and ionomycin will activate all cells, including T cells in an antigen independent manner, whereas antigen-specific stimulation will only activate antigen-specific T cells. Therefore, the increased number of IFNγ-producing T cells are not likely to be *H. polygyrus* dependent, and are an artefact of unspecific PMA and ionomycin stimulation. *H. polygyrus* antigen-specific Th2 recall responses were also intact in *Pla2g1b*$^{-/-}$ mice, with similar amounts of IL-13 and IL-5 produced (**Figure 3.6.E**). Treg cells, which can be induced by *H. polygyrus* secretory products (Grainger et al., 2010) and are capable of suppressing Th2 cell-mediated inflammation (Wilson et al., 2005), were also unaffected by *Pla2g1b*-

deficiency (**Figure 3.6.F-G**). Taken together, these data indicate that there were no qualitative and only minor quantitative differences in T cell responses and T cell-derived cytokines following 2^{o} *H. polygyrus* infection.

3.2.4.2 *Pla2g1b* is not required for appropriate antibody response to *H. polygyrus*

B cell class switching and antibody production is essential for protective immunity to *H. polygyrus* infection (Liu et al., 2010, McCoy et al., 2008). *Pla2g1b*-deficiency did not affect total CD19^{+} B cell frequencies at steady state (**Figure 3.4.G**) and similarly IgE or *H. polygyrus*-specific IgG1 production was not altered upon 2^{o} *H. polygyrus* infection in *Pla2g1b*$^{-/-}$ mice (**Figure 3.7.A-C**). These data illustrate that PLA_2g1B is not required for peripheral B cell frequencies or function.

3.2.4.3 *Pla2g1b*-deficiency does not alter alternative activation of macrophages

Stimulation through the IL-4Rα, a subunit of the IL-4 receptor used by both IL-4 and IL-13, promotes alternative activation of macrophages (Goerdt and Orfanos, 1999). In 2^{o} *H. polygyrus* infection, aaMϕs are important essential for protective immunity to *H. polygyrus* (Anthony et al., 2006). Arginase 1 (*Arg1*), Relmα (*Retnla*) and Ym1 *(Chi3l3*), gene markers of alternative activation of macrophages (Rodriguez-Sosa et al., 2002, Dasgupta et al., 2011, Raes et al., 2002), were intact in the small intestine of *Pla2g1b*$^{-/-}$ mice upon 2^{o} infection (**Figure 3.8.A**). Similarly, macrophage intrinsic *Pla2g1b* was confirmed to not be required for alternative activation of macrophages *in vitro*, with IL-4 and IL-13 stimulation of BMDMs inducing expression of Arginase 1 (*Arg1*), Relmα (*Retnla*) and Chitiase-like 3 *(Chi3l3*) in both WT and *Pla2g1b*-deficient macrophages (**Figure 3.8.B**). These data collectively demonstrate that aaMϕs were intact in *Pla2g1b*$^{-/-}$ mice.

3.2.4.4 ILC2 populations are unaffected in *Pla2g1b*$^{-/-}$ mice following *H. polygyrus* infection

Previous reports have illustrated that ILC2s expand in number following *H. polygyrus* infection and are important in supporting early Th2 differentiation (Pelly et al., 2016). We therefore set out to identify whether *Pla2g1b* deficiency affected the ILC2 population. As was demonstrated in naïve *Pla2g1b*$^{-/-}$ mice (**Figure 3.4.H**), the ILC2 population was also unaffected in the mLN following 2^{o} *H. polygyrus* infection (**Figure 3.9**).

Taken together, type 2 immune responses appeared to be fully intact in *Pla2g1b*-deificent mice. Despite this, mice lacking *Pla2g1b* failed to expel a 2^{o} *H. polygyrus* challenge infection. We next set out to determine the function of PLA_2g1B in *H. polygyrus* infection, beyond type 2 immune responses.

3.2.5 *Pla2g1b*-deficient mice display normal lipid metabolism and generation of bioactive lipids

PLA_2 enzymes function by hydrolysing phospholipids into fatty acids and lysophospholipids, with many of these products often biologically active with important downstream roles (Six and Dennis, 2000). PLA_2g1B has primarily been described as a dietary phospholipase, secreted from the pancreas, responsible for digesting dietary fats to allow for their absorption (Richmond and Hui, 2000). However, at steady state, *Pla2g1b*$^{-/-}$ mice were shown to have no defect in dietary fat digestion and absorption, with defects only seen upon administration of a high fat diet (Richmond et al., 2001, Labonte et al., 2010, Labonte et al., 2006). To determine whether PLA_2g1B-mediated dietary phospholipid digestion was altered in *Pla2g1b*$^{-/-}$ mice upon 2^{o} *H. polygyrus* infection, we measured serum metabolites from WT and *Pla2g1b*$^{-/-}$ mice. No significant difference in serum lipid metabolites, triglycerides, glycerol, free fatty acids or LPC in naïve or infected mice were observed between WT and *Pla2g1b*$^{-/-}$ mice (**Figure 3.10.A-B**), suggesting that there was no defect in lipid digestion and absorption. There was a significant decrease in LPC concentration in the small intestinal homogenate of naïve *Pla2g1b*$^{-/-}$ mice, which was unchanged upon 2^{o} infection (**Figure 3.10.B**). However, in WT mice, the increased LPC

concentration was reduced to the same concentration as the *Pla2g1b*$^{-/-}$ mice upon 2^{o} *H. polygyrus* infection (**Figure 3.10.B**). The difference in small intestinal LPC in naïve mice did not translate to differences in serum LPC concentration.

sPLA$_2$ enzymes, including PLA$_2$g1B, are also capable of activating intracellular cPLA$_2$ via binding the sPLA$_2$R (Fonteh et al., 1998, Fonteh et al., 2000), hydrolysing phospholipids in the cell membrane to release arachidonic acid, initiating the eicosanoid production pathway (Funk, 2001). Eicosanoids are both induced and important in immunity to intestinal helminth infection (Douch et al., 1996, Machado et al., 2005). However, no defect in the production of eicosanoids, specifically in cysteinyl leukotriene or prostaglandin synthesis, was observed in small intestinal homogenate from 2^{o} *H. polygyrus* infected *Pla2g1b*$^{-/-}$ mice (**Figure 3.10.C**).

Overall, these data illustrate that *Pla2g1b*-deficiency does not affect dietary lipid digestion and absorption or eicosanoid production in *H. polygyrus* infection.

3.2.6 No gross transcriptomic differences were identified in *Pla2g1b*-deficient mice at baseline or upon 2^{o} *H. polygyrus* infection

In an attempt to identify the mechanism by which PLA$_2$g1B mediates resistance to *H. polygyrus* infection, we harvested duodenal tissue from naïve and 2^{o} *H. polygyrus* infected (7 days-post infection) WT and *Pla2g1b*$^{-/-}$ mice. Following RNA extraction and RNA sequencing, we could not identify any differences in the transcriptional landscape between genotypes in naïve mice, with the exception of *Pla2g1b* (**Figure 3.11.A**). In 2^{o} infected mice, we saw decreased expression of both *Pla2g1b* and *Lars2* in *Pla2g1b*$^{-/-}$ mice, however the downregulation of *Lars2* did not reach statistical significance (FDR = 0.058, relative to WT *H.p.* 2^{o}) (**Figure 3.11.A**). Pathway analysis confirmed the similarity of the two genotypes upon 2^{o} *H. polygyrus* infection, with predicted activation of immune and inflammatory pathways evident (**Figure 3.11.B**). We also saw no defect in the predicted activation of lipid metabolism pathways in *Pla2g1b*$^{-/-}$ mice upon 2^{o} infection (**Figure 3.11.C**), supporting the conclusions drawn from the data presented above in **3.2.5** (**Figure 3.10**). RNA sequencing data identified similar levels of goblet cell and mucus-associated genes, which correlate with expulsion (Hashimoto et al., 2009, Hasnain et al., 2010, Inagaki-

Ohara et al., 2011). Similarly, AB-PAS staining intestinal tissue appeared comparable between WT and *Pla2g1b*$^{-/-}$ mice (**Figure 3.11.D-E**). The increased expression of the goblet cell-derived antihelminth molecule Relmβ *(Retnlb*), which inhibits adult *H. polygyrus* feeding *in vivo* (Herbert et al., 2009), was also unaffected in *Pla2g1b*-deficient mice (**Figure 3.11.D**). All other PLA_2 enzymes expressed in the small intestine were not altered by the absence of *Pla2g1b* (**Figure 3.11.F**). These data provide one explanation why lipid metabolism pathways (**Figure 3.11.C**), lipid metabolite uptake and eicosanoid production were unaffected in *Pla2g1b*$^{-/-}$ mice (**Figure 3.10**). Overall, these data illustrate that *Pla2g1b*-deficiency does not affect the transcriptional response to 2^{o} *H. polygyrus* infection, suggesting that PLA_2g1B is not acting like a 'cytokine' or second messenger in antihelminth immune responses.

3.2.7 PLA_2g1B has direct anthelmintic properties which act in synergy with the immune system to trap and kill *H. polygyrus*

3.2.7.1 PLA_2g1B treatment compromises *H. polygyrus* establishment *in vivo*

Previous reports have demonstrated essential roles of $sPLA_2$ enzymes in defence against fungal infections (Balestrieri et al., 2009) and in providing bactericidal activity through bacterial membrane degradation (Weinrauch et al., 1998, Degousee et al., 2002, Koduri et al., 2002). We therefore asked whether PLA_2g1B had a direct effect on *H. polygyrus*, promoting resistance to infection.

To test this, we treated exsheathed L3 *H. polygyrus* larvae (to recapitulate the status of the larvae after passing through the stomach (Sommerville, 1957, Sommerville and Bailey, 1973)) with recombinant mouse PLA_2g1B for 24 hours *in vitro*, before infecting naïve WT mice. Following infection with the PLA_2g1B-treated larvae, we saw no defect in the ability of the larvae to migrate to the small intestine and embed into the intestinal wall (**Figure 3.12.A**), suggesting PLA_2g1b treatment does not perturb infectivity of L3 *H. polygyrus* larvae. However, we identified a dose-dependent reduction of luminal L5 adult *H. polygyrus* worms 14 days-post infection, with a significant reduction seen with both 1 and 10 ng/µL PLA_2g1B-treated larvae. (**Figure 3.12.B**). PLA_2g1B-mediated protection was abrogated when PLA_2g1B was heat inactivated or treated with the PLA_2 inhibitor manoalide (**Figure 3.12.B-C**),

illustrating that the catalytic activity of PLA_2g1B was essential in mediating its anthelmintic effect. The significant reduction in adult worms did not translate into reduced *H. polygyrus* egg production, with a non-significant decline seen after 10 ng/µL PLA_2g1B treatment (**Figure 3.12.D**). These data collectively suggest that *in vitro* PLA_2g1B treatment of infective L3 *H. polygyrus* larvae has direct anthelmintic effects; perturbing larval development and/or rendering the larvae more susceptible to killing in the wall of the small intestine

3.2.7.2 PLA_2g1B treatment does not compromise the fitness of *H. polygyrus in vitro* or *ex vivo*

To further investigate PLA_2g1B effects on *H. polygyrus*, we treated L3 larvae, sheathed or exsheathed, and L5 adult worms *in vitro* with PLA_2g1B for 24 hours, before measuring ATP concentration in the worms, an assay previously used to determine helminth fitness (Ishiwata and Watanabe, 2007, Herbert et al., 2009) (**Figure 3.13.A**). Following PLA_2g1B treatment, no significant difference was seen in ATP concentration of sheathed or exsheathed L3 larvae (**Figure 3.13.B**). Similarly, no significant difference in L5 adult worm fitness was identified following PLA_2g1B treatment (**Figure 3.13.C**). We then asked whether *in vitro* PLA_2g1B treatment compromised the fitness of *H. polygyrus in vivo*. To test this, we treated exsheathed L3 larvae *in vitro* and infected WT mice. We then isolated *H. polygyrus* at either the L4 or L5 stage of development and analysed their fitness *ex vivo*, determined by ATP concentration (**Figure 3.13.D**). Isolation of L4 or L5 adult *H.* polygyrus, 7 or 14 days-post infection respectively, revealed a decrease in ATP concentration following PLA_2g1B treatment, although not significant in either developmental stage (**Figure 3.13.E-F**). Overall, the *in vitro* data suggest that PLA_2g1B does not compromise *H. polygyrus* fitness alone, but requires another factor, such as environment-induced moulting or other antihelminth immune pressures, found *in vivo,* to impact fitness and development.

3.2.7.3 PLA$_2$g1B and the immune compartment act in synergy to provide protect against intestinal helminth infection

Following the observation that PLA$_2$g1B did not have a significant impact on worm ATP levels (section **3.2.7.2**, above) and that the protective effect of PLA$_2$g1B-treated L3 larvae (**Figure 3.12**) did not recapitulate the full clearance of worms observed during 2^o *H. polygyrus* infection in WT mice (**Figure 3.5.A**), we hypothesised that for complete elimination of *H. polygyrus*, a combined PLA$_2$g1B-mediated effect on larvae alongside immune-mediated physiological responses would be required. To test this and test whether direct PLA$_2$g1B treatment could rescue immunity in *Pla2g1b*$^{-/-}$ mice, we 1^o infected and drug-cured WT and *Pla2g1b*$^{-/-}$ mice to elicit robust type 2 immune responses (as reported in section **3.2.4**) and performed 2^o challenge infection with vehicle- or PLA$_2$g1B-treated L3 larvae (**Figure 3.14.A**). As expected, WT mice killed the majority of both vehicle- and PLA$_2$g1B treated *H. polygyrus* larvae and *Pla2g1b*$^{-/-}$ mice failed to clear vehicle-treated larvae (**Figure 3.5.A**). However, full *H. polygyrus* killing and clearance was fully rescued in *Pla2g1b*$^{-/-}$ mice when the challenge bolus of L3 larvae were treated with PLA$_2$g1B (**Figure 3.14.B**).

To further test the requirement for the cooperation of a functional immune compartment and direct PLA$_2$g1B effects, we infected *Rag*$^{-/-}$*γc*$^{-/-}$ mice with PLA$_2$g1B-treated larvae (**Figure 3.14.A**). Following 1^o infection, WT mice that were infected with PLA$_2$g1B-treated larvae had a reduced worm burden 14 days-post infection, as expected (**Figure 3.12**, **Figure 3.14.C**). However, *Rag*$^{-/-}$*γc*$^{-/-}$ mice 1^o infected with PLA$_2$g1B-treated larvae were unable to kill and expel *H .polygyrus*, with no significant difference observed from vehicle-treated *H. polygyrus* larvae (**Figure 3.14.C**). PLA$_2$g1b-treatment of L3 larvae was also unable to rescue *H. polygyrus* killing and clearance in *Rag*$^{-/-}$*γc*$^{-/-}$ mice following 2^o infection (**Figure 3.14.D**).

Overall, these data presented demonstrate that direct PLA$_2$g1B-mediated anthelmintic effect acts in synergy with a functional immune compartment, most likely the type 2 immune response, to induce full killing and resistance to *H. polygyrus*.

3.2.8 PLA_2g1B-treatment induces changes in L3 larval lipids

3.2.8.1 PLA_2g1B induces no overt structural changes to L3 *H. polygyrus* larvae

We hypothesised that PLA_2g1B may mediate its direct anthelmintic actions on *H. polygyrus* by inducing physical damage and/or structural changes to L3 larvae, perturbing normal development *in vivo*. To test this, we performed scanning electron microscopy on vehicle and PLA_2g1B-treated exsheathed L3 *H. polygyrus* larvae. No gross physical changes were observed following PLA_2g1B treatment of L3 larvae, with the anterior, midriff and posterior structure appearing fully intact (**Figure 3.15**).

3.2.8.2 PLA_2g1B-treatment induces a lower abundance of phospholipids in L3 larvae.

PLA_2 enzymes hydrolyse phospholipids (Kudo and Murakami, 2002). We therefore asked whether PLA_2g1B altered the phospholipid composition of L3 *H. polygyrus* larvae. To test this, we extracted lipids from vehicle- or PLA_2g1B-treated L3 larvae and analysed the lipid composition using LC-MS/MS. Of the 1165 apolar features detected, 112 were significantly different between the chromatograms of vehicle- and PLA_2g1B-treated larvae, identifying significantly lower phospholipid abundance in PLA_2g1B-treated larvae (**Figure 3.16.A**). Of the 112 significant apolar features, 6 were identified as phosphatidylethanolamines (PEs) by comparing their precursor ion and MS/MS fragments with the LipidBlast library (**Figure 3.16.B-E**), with each being lower in PLA_2g1B-treated larvae (**Figure 3.17.A**).

PEs consist of a phosphoethanolamine head group attached to a glycerol backbone, itself attached to two fatty acid moieties via phosphoether and ester bonds, respectively (van Meer et al., 2008). As a result, PE MS spectra display traits of fatty acid-containing molecules; clusters that have inter-cluster mass shifts of 28 Da (CH_2CH_2) and intra-cluster mass shifts of 2 Da, indicative of difference in double bond number (*i.e.* fatty acid saturation) (**Figure 3.16.B**). From this, we identified and annotated the ions of PE species using positive-ion MS/MS and negative-ion fragmentation, quantified in **Figure 3.17.A**. For example, we show the identification

of PE 36:3 (18:1, 18:2), which is reduced in abundance in PLA_2g1B-treated larvae (**Figure 3.16.C**, **Figure 3.17.A**). Positive ion MS/MS of PE 36:3 (18:1, 18:2) was used to confirm assignment of the peak as PE, where neutral loss of 141 Da indicates a phosphoethanolamine head group, fragmentation indicated on structural diagram (**Figure 3.16.D**). Negative ion fragmentation of the ion PE 36:3 (18:1, 18:2) identified both fatty moieties as 18:1 and 18:2, fragmentation indicated on structural diagram (**Figure 3.16.E**). However, both the arrangement of the fatty acid moieties at the glycerol backbone (i.e. *sn*-1 or *sn*-2) and the position of the double bonds could not be inferred from this analysis.

A further 3 features could be putatively annotated as PEs by using other identified PEs as a reference; comparing peak retention time, inter-cluster mass shifts of 28 Da (CH_2CH_2) and intra-cluster mass shifts of 2 Da. However, MS/MS could not be performed due to low abundance, preventing confirmation of PE identity. These three putatively annotated PEs were, again, lower in abundance in PLA_2g1B-treated larvae (**Figure 3.17.B**). The remaining significantly different apolar features were unable to be identified, possessing lipidic features that could not be annotated, even putatively, by comparison to common libraries (**Figure 3.17.C**). These unidentifiable apolar features were seen to both increase and decrease in abundance following PLA_2g1B treatment (**Figure 3.17.C**).

Overall these data suggest that PLA_2g1B is cleaving phospholipids present in *H. polygyrus* L3 larvae. Whether this compromises larval development or promotes host resistance to invading larvae is still to be determined.

3.2.9 Intestinal *Pla2g1b* upregulation requires drug-clearance of primary *H. polygyrus* infection

Our initial RNA sequencing data demonstrated that *Pla2g1b* expression was upregulated in the small intestine of drug-treated resistant mice, with or without 2^o challenge infection, up to 49 days-post drug treatment (**Figure 3.3.C**). We therefore investigated the kinetics of *Pla2g1b* upregulation in more detail within our 2^o challenge infection model. As *Pla2g1b* is abundantly expressed in the pancreas (Eerola et al., 2006), we initially tested whether pancreatic *Pla2g1b* expression was

altered in our infection model. No change in *Pla2g1b* expression in the pancreas throughout our model was observed (**Figure 3.18.A**). However, we confirmed upregulation of *Pla2g1b* in the duodenal tissue of resistant mice, with significant upregulation seen at day 28, 14 days-post drug-treatment (**Figure 3.18.B**). Upregulation of *Pla2g1b* was not seen during the course of a 1^{o} infection (**Figure 3.18.C**), or with drug-treatment alone in the absence of a 1^{o} infection (**Figure 3.18.D**). Upregulation of *Pla2g1b* required drug-clearance of a 1^{o} infection (**Figure 3.18.D**). These data indicate that *Pla2g1b* expression is elevated in the small intestine concomitantly with the acquisition of resistance to *H. polygyrus*. We next set out to determine both the source of *Pla2g1b* expression in the small intestine.

3.2.10 *Pla2g1b* expression is restricted to epithelial cells in the small intestine of resistant mice.

The family of sPLA$_2$ enzymes share a high degree of homology, making it challenging to raise specific antibodies against PLA2g1B. This problem, coupled with the fact that PLA$_2$g1B is a secreted protein, make it technically very challenging to perform immunohistochemistry to identify the source of PLA$_2$g1B in the small intestine. We therefore utilised *in situ hybridisation* technology to identify the cellular source of *Pla2g1b* expression in small intestinal tissue of resistant mice. *In situ hybridisation* identified that *Pla2g1b* expression was restricted to the epithelial layer of resistant mice (**Figure 3.19.A-B**), and not present in the granuloma surrounding the encysted larvae, 7 days-post 2^{o} infection (**Figure 3.19.C**). To confirm *Pla2g1b* expression was expressed in the intestinal epithelial cells of resistant mice we utilised FACS to isolate epithelial cells from infected mice. However, due to technical difficulties in obtaining viable cells when sorting from the intestine during 2^{o} challenge infection, we sorted cells from the intestine of resistant mice at day 28, 14 days after drug-cure, when *Pla2g1b* expression was significantly increased (**Figure 3.18.B**). CD45^{-}EpCam^{+} epithelial cells sorted from resistant mice had significantly increased *Pla2g1b* expression, compared to naïve mice, whereas there was no change in CD45^{+}EpCam^{-} sorted cells (**Figure 3.19.D**). The FACS data corroborates the *in situ hybridisation* data, confirming that *Pla2g1b* expression was increased in resistant mice and expressed in the epithelial cells of the small intestine.

3.2.11 Regulation of *Pla2g1b*

3.2.11.1 *Pla2g1b* expression is regulated by the microbiota and Rag- and common gamma chain-dependent immune cells *in vivo*

IL-4 is necessary and sufficient for resistance to *H. polygyrus* (Urban et al., 1991b). We therefore asked whether IL-4 was required, directly or indirectly, for induction of *Pla2g1b* expression in resistant mice. To test this, we treated mice with anti-IL-4 antibody (αIL-4) or an isotype control before, during and after drug-clearance of 1° infection and then analysed *Pla2g1b* expression in the duodenal tissue at day 28, 14 days after drug-cure. Interestingly, αIL-4 treatment had no effect on intestinal *Pla2g1b* expression in resistant mice (**Figure 3.20.A**), suggesting that *Pla2g1b* is not regulated in an IL-4 or type-2 immune dependent manner. To test whether Rag-dependent adaptive immune cells or common gamma chain-dependent innate immune cells were required for *Pla2g1b* expression in resistant mice, we infected and drug-cured *Rag*$^{-/-}$*γc*$^{-/-}$ mice and measured *Pla2g1b* expression in the small intestine by qRT-PCR. *Pla2g1b* upregulation was abrogated in *Rag*$^{-/-}$*γc*$^{-/-}$ resistant mice (**Figure 3.20.B**), suggesting that adaptive or innate immune cells or *γc*-dependent signalling is required to induce *Pla2g1b* expression in intestinal epithelial cells of resistant mice.

To identify additional type-2 independent mechanisms of *Pla2g1b* upregulation, we asked whether the host microbiota contributed to *Pla2g1b* regulation. Intestinal helminth infection has previously been described to induce changes in the composition of the intestinal microbiota (Rausch et al., 2013, Reynolds et al., 2014b, Zaiss et al., 2015, Giacomin et al., 2016, Kannan et al., 2017), however further changes following drug-cure induced resistance have not been studied. To test whether the intestinal microbiota was required for the upregulation of *Pla2g1b*, we treated mice with a cocktail of antibiotics prior to (day -7) and all throughout 1° infection and drug-cure until harvest at day 28. Antibiotic-treated mice completely failed to upregulate *Pla2g1b* (**Figure 3.20.C**), indicating that intestinal microbiota are essential for elevated *Pla2g1b* expression in resistant mice. Following on from this observation, we next tested if antibiotic treatment would perturb resistance,

preventing clearance of 2º *H. polygyrus* challenge infection and, if so, could PLA_2g1B treatment of the infective L3 larvae restore immunity. Indeed, we identified that antibiotic treatment, prior to (day -7) and all throughout 1º infection, drug-cure and 2º infection, prevented expulsion of 2º challenge infection (**Figure 3.20.D**). Furthermore, the number of luminal worms in the antibiotic-treated mice was comparable to that of a 1º infection (infective dose 64 adult worms, data not shown). However, PLA_2g1B treatment of the L3 infective larvae did not rescue killing and clearance of 2º *H. polygyrus* infection in antibiotic-treated mice (**Figure 3.20.D**).

Taken together, these data indicate that upregulation of *Pla2g1b* expression in the epithelial cells of resistant mice requires both the microbiota and Rag- and/or common gamma chain-dependent immune cells or signalling. In addition, antibiotic treatment abrogates protective immunity to *H. polygyrus* which cannot be rescued by PLA_2g1B treatment of the L3 larvae prior to infection.

3.2.11.2 *Pla2g1b* expression does not correlate with goblet and tuft cell marker expression

With *Pla2g1b* expression restricted to and upregulated in intestinal epithelial cells of resistant mice (**Figure 3.19**), we utilised the intestinal organoids culture system to determine how *Pla2g1b* expression was regulated. Intestinal organoid culture is an *in vitro* culture system of intestinal epithelial stem cells which differentiate into all epithelial cell types and self-organise in crypt-villus structures in the absence of a non-epithelial cellular niche (Sato et al., 2009). Upon stimulation with IL-4 and IL-13, we identified a significant downregulation of *Pla2g1b* expression and upregulation of the goblet cell markers Relmβ (*Retnlb*) and *Gob5* (**Figure 3.21.A**). These data suggest that *Pla2g1b* is negatively regulated by IL-4Rα signalling in the *in vitro* intestinal organoid system, contrasting to the *in vivo* data where blocking IL-4 had no effect on *Pla2g1b* expression. However, the organoid data could also be interpreted that *Pla2g1b* expression is not located in the epithelial cell subtypes induced by IL-4Rα signalling, both tuft and goblet cells (Gerbe et al., 2016). The small intestine RNA sequencing data set (see section **3.2.**1) demonstrates that tuft cell gene markers, *Dclk1*, *Trpm5*, *Pou2f3* and *Siglec5 (Gerbe et al., 2016, von Moltke et al.,*

2016, Howitt et al., 2016), do not correlate with *Pla2g1b* expression (**Figure 3.3.C**, **Figure 3.21.B**).

In an attempt to identify factors which induce *Pla2g1b* expression in the intestinal organoid culture system, we treated organoids with small intestinal homogenate from resistant mice following 2º infection. Intestinal homogenate from resistant mice was able to drive *Pla2g1b* expression in intestinal organoids, although not reaching statistical significance (p=0.088) (**Figure 3.21.A**). This data suggests that the factor(s) to drive *Pla2g1b* expression are present in resistant mouse intestine or lumen, however further experiments are required to conclusively demonstrate this; perhaps by titrating or fractionating the duodenal homogenate from resistant mice.

Further investigation of intestinal epithelial cell subtypes maybe required to determine the precise source of *Pla2g1b* expression. Moreover, identification of the critical factor in the small intestinal homogenate would provide greater understanding of how *Pla2g1b* is expressed in intestinal epithelial cells.

3.3 Discussion

Intestinal helminths have evolved with their hosts over thousands of years (Cox, 2002) and, as a result, are highly effective in establishing chronic infections. Although host mortality is rare, host morbidity is common. Human soil transmitted helminths are highly prevalent in developing countries (Bethony et al., 2006, Hotez et al., 2008). With the failing of vaccine efforts, limited numbers of chemotherapeutic drugs and the emergence of drug-resistant helminths (Albonico et al., 2003, Taman and Azab, 2014) there is a need for a better understanding of antihelminth immunity and generation of new therapeutics.

To address this issue, we set out to provide a global analysis of the small intestinal transcriptome during *H. polygyrus* 1º and 2º infection. *H. polygyrus*, as a model of human helminthiasis, allowed us to compare susceptibility and acquired resistance, in the same genetic background of mice, to identify critical regulators of immunity. From this analysis, we identified that *Pla2g1b* expression was upregulated in resistant mice, with or without 2º infection. Furthermore, PLA_2g1B had direct anthelmintic effects and was essential for resistance to *H. polygyrus*. The remainder

of this chapter discusses our findings in in the context of antihelminth immunity, highlighting implications, shortfalls and further questions.

3.3.1 RNA sequencing as a tool to identify mechanisms of antihelminth immunity

RNA sequencing of duodenal tissue from C57BL/6 mice identified many genes previously implicated in resistance to *H. polygyrus* infection, including Arginase 1 (*Arg1*) (Anthony et al., 2006) and Relmβ (*Retnlb*) (Herbert et al., 2009). Interestingly, these genes were among those we identified that were quantitatively different between 1^{o} (susceptible) and 2^{o} (resistant) mice, detailed in Cluster 1 (**Figure 3.1.E**, **Table 3.1**). This pattern of expression was mimicked in the pathways analysis, where 'diseases and biological function pathways' were predicted to be activated in both 1^{o} and 2^{o} infection but to a greater magnitude in 2^{o} infection (**Figure 3.2.A**). These observations support the prevailing notion of resistance to *H. polygyrus*; trapping and killing of invading larvae is promoted by a memory type 2 immune response, inducing a faster and stronger immune response upon infection (Morimoto et al., 2004, Pelly et al., 2016, Pelly et al., 2017).

Accompanying these quantitative differences, we also identified qualitative differences between susceptible and resistant mice, with gene signatures only present in resistant mice. The number of unique, differentially expressed genes was 4.5 times greater in *H.p.* 2^{o} than in *H.p.* 1^{o} (relative to naïve, 2-fold filter, p<0.05) (**Figure 3.1.D**). These *H.p.* 2^{o}-unique genes were separated into two distinct clusters, based upon upregulation (Cluster 2, **Table 3.2**) or down-regulation (Cluster 3, **Table 3.3, Figure 3.1.E**). The majority of these genes had not previously been described in resistance to intestinal helminth infection. We also identified a significant increase in predicted activation of lipid metabolism pathways during 2^{o} infection (**Figure 3.3.A-B**), leading us to investigate *Pla2g1b* during resistance to intestinal helminth infection.

The design of our RNA sequencing experiment incorporated additional groups, enabling the capacity for more detailed analysis of susceptible and resistant mice. These additional groups included resistant mice that were not 2^{o} challenge infected

after drug clearance of a 1^{o} infection (Rx (D42)). Additionally, we also incorporated groups which were left a longer period of time after drug clearance of 1^{o} infection before receiving 2^{o} infection (*H.p.* 2^{o} (D63)) or not (Rx (D63)) (**Figure 3.1.A**). The incorporation of *H.p.* 2^{o} (D63) allowed us to confirm that genes identified in *H.p.*2^{o} (D42) were also present during 2^{o} challenge infection at a later time, suggesting that that they are essential for long term resistance to *H. polygyrus* infection. The addition of groups Rx (D42) and Rx (D63) gave us the ability to identify any changes in the transcriptome of the small intestine after resolution of a 1^{o} infection, that were maintained over a long time period (**Figure 3.2.C**). These time points also reduced the likelihood of identifying acute tissue repair genes and instead suggested a 'new baseline', promoting resistance to subsequent 2^{o} challenge infection. As a result, we identified that *Pla2g1b* expression in the small intestine was upregulated following drug-mediated clearance of 1^{o} infection and maintained for up to 49 days, with or without 2^{o} infection (**Figure 3.3.C**) and correlated with resistance.

Although not performed here, it would be of great interest to study the intestinal transcriptome of groups Rx (D42) and Rx (D63), relative to naïve mice. This would enable description of the new transcriptional baseline of resistant mice and potentially identify other genes, previously undescribed in antihelminth immunity, essential for protection against 2^{o} *H. polygyrus* challenge infection. This is an area that is particularly understudied in antihelminth immunity, with the majority of research focussed on the immune response during infection. This research would be extremely informative and potentially aid effective antihelminth vaccine efforts.

3.3.2 Anthelmintic properties of PLA$_2$g1B

3.3.2.1 A new role for an 'old' sPLA$_2$

PLA$_2$g1B was one of the first sPLA$_2$ enzymes discovered and studied in mammals, originally purified from the pancreas (Murakami et al., 2014). PLA$_2$g1B was originally described as a digestive sPLA$_2$, secreted from the pancreas into the intestinal lumen following a high fat meal, responsible for cleaving dietary and biliary phospholipids (Murakami et al., 2014, Murakami et al., 2015, Williams et al., 1989, Richmond et al., 2001). Perturbation of PLA$_2$g1B activity protects from diet-induced obesity,

hyperglycaemia, insulin resistance, hepatic steatosis and atherosclerosis due to reduced intestinal absorption of lipids (Labonte et al., 2006, Hui et al., 2009, Labonte et al., 2010, Hollie and Hui, 2011, Hollie et al., 2014, Hui, 2016).

Here we demonstrate an essential role for PLA_2g1b in resistance to intestinal helminth infection (**Figure 3.5**), which appears to be independent of dietary lipid absorption and generation of bioactive lipid mediators (**Figure 3.10**). Interestingly, we did identify a significant decrease in LPC concentration in the small intestinal homogenate of WT mice upon 2º *H. polygyrus* infection, compared to naïve tissue. This decrease was absent in *Pla2g1b*$^{-/-}$ mice, which maintained a significantly lower LPC concentration in both naïve and 2º infected tissue (**Figure 3.10.B**). These data suggest that LPC may be metabolised locally following *H. polygyrus* infection in WT mice, a process that is absent in *Pla2g1b*$^{-/-}$ mice. Whether intestinal PLA_2g1B-derived LPC is essential for mediating resistance to 2º challenge infection, however, is unclear. However, it is unlikely any PLA_2g1B-induced dietary lipids contribute to intestinal immunity, including LPC, as critical antihelminth immune components are intact in naïve and infected mice (See section **3.2.2** and **3.2.4**), although we cannot rule out any other direct or indirect effects of dietary lipids in antihelminth immunity.

Instead we show that PLA_2g1B had direct anthelmintic effects on *H. polygyrus* L3 larvae, reducing global phospholipid abundance and specifically reducing PE species (**Figure 3.16**, **Figure 3.17**). Although both human and murine PLA_2g1B have direct antibacterial properties *in vitro*, albeit significantly less potent than other $sPLA_2$ family enzymes (Koduri et al., 2002), this is the first time PLA_2g1B has been shown to be protective against an infective pathogen *in vivo*. Despite other PLA_2 enzymes implicated in protective immunity to intestinal helminths, particularly $cPLA_2$ enzymes in the generation of LTs and other downstream mediators (Brown et al., 2008, Patnode et al., 2014, Rogerio and Anibal, 2012, Espinoza et al., 2002), their role has never been shown to be essential. Furthermore, no other $sPLA_2$ enzyme has been shown to have direct action on intestinal helminths.

3.3.2.2 Role of phospholipids in intestinal helminths

Using LC-MS/MS, we were able to identify that PLA_2g1B treatment reduced the global phospholipid abundance of L3 *H. polygyrus* larvae (**Figure 3.16**). Specifically, we were able to and putatively identify and confirm the identity of PEs, which were reduced following PLA_2g1B treatment (**Figure 3.16** and **Figure 3.17**). Unfortunately, we were not able to identify other lipidic compounds due to low abundance, however we confirmed that these compound are likely to be lipids due to their apolar features. Further optimisation of the lipid extraction method and increasing our starting material may be required to gain a greater resolution in our data analysis.

Phospholipids are an essential component in all cell membranes, composed of two hydrophobic fatty acids tails and a hydrophilic phosphate head group connected by a glycerol molecule (van Meer et al., 2008). The phosphate groups contain different molecules, such as choline or serine, thus providing the phospholipids with different properties. Helminth parasites possess phospholipids in their membranes, containing known and unknown phospholipid species, differing between helminths (Retra et al., 2015, Van Hellemond et al., 2006, Satouchi et al., 1993, Beames and Fisher, 1964, Beames, 1964).

PE is a highly abundant phospholipid, present in membranes of mammals, bacteria, yeast and intestinal nematodes (Beames, 1964), and is required for an array of cellular functions, acting both as a signalling molecule and a precursor for signalling molecules (Vance and Tasseva, 2013). Roles for PE have been described in membrane curvature, cellular stress, mitochondrial morphology as well as brain and liver function (Vance and Tasseva, 2013).

The role of PE, and other phospholipid species, are not well described in intestinal helminths. However, in the free-living nematode, *C. elegans*, low levels of PE in the brain lead to endoplasmic reticulum stress and neurodegeneration, indicating that PE is essential for brain function (Wang et al., 2014).

Further work is required to understand the role of phospholipids in intestinal helminths, with work so far providing a description of phospholipid content and diversity in intestinal helminths. Until genetically modifiable intestinal helminth tools are available, this line of research will be particularly challenging. The use of

synthetic, and possibly more potent, phospholipases may be of use to test the requirement of phospholipids in *H. polygyrus* viability. Additionally, the use of *C. elegans* as a model organism may also provide clues to the functions of phospholipids in infective helminths.

3.3.2.3 Endogenous anthelmintic molecules

Despite a good understanding of the protective type 2 immune response to intestinal helminth infection (Allen and Maizels, 2011, Reynolds et al., 2012, Maizels et al., 2012b), we do not yet know the precise mechanism how invading helminths are killed. Host-derived endogenous antihelminth molecules have been described, including Relmβ and Arginase. These add to the arsenal of other endogenous antimicrobial proteins, such as cathelicidins, defensins, histatins, lysozyme and lactoferrin (Zelechowska et al., 2016).

Relmβ is the only molecule previously described to have direct effects on intestinal helminths, essential for immunity to lumen-dwelling small intestinal helminths. Herbert and colleagues elegantly demonstrate that Relmβ impairs adult *H. polygyrus* feeding on host tissue *in vivo* (Herbert et al., 2009). Arginase production from aaMϕ is essential for the trapping of *H. polygyrus* larvae in the intestinal wall, impairing larval health and preventing emergence of adult worms into the lumen (Anthony et al., 2006). The authors did not treat intestinal larvae with recombinant Arginase *in vitro* to determine whether Arginase has direct anthelmintic properties, although they suggest it is not due to Arginase- or aaMϕ-dependent collagen deposition trapping of the larvae, as collagen was only detected 12 days-post 2^{o} *H. polygyrus* infection (Anthony et al., 2006).

Endogenous anthelmintics have been suggested to be present in the small intestine of sheep resistant to intestinal helminth infection. Douch and colleagues demonstrated that compounds present in the gastrointestinal mucus of nematode resistant sheep had antihelminth activity (Douch et al., 1996). Sheep were repeatedly infected and drug-treated with *Trichostrongylus colubriformis* or *Haemonchus contortus* L3 larvae. Small intestinal sections were then ligatured to form sacs that were then incubated with exsheathed L3 larvae for 1 hour. The L3 larvae that were

incubated in resistant sheep small intestinal tissue were significantly less motile than those incubated in uninfected sheep small intestine (Douch et al., 1996). The authors suggested the antihelminth activity of the resistant small intestine mucosa was due to LTs and other 'anti-parasitic' substances, however these were not formally tested in this study. Others have suggested that LTs have a role in antihelminth immunity, however direct effects on intestinal helminths have not been confirmed (Moqbel et al., 1990, Machado et al., 2005). If LTs are essential for killing of *H. polygyrus*, they may act in concert with PLA_2g1B as cLTs were intact in *Pla2g1b*$^{-/-}$ mice, which failed to expel *H. polygyrus* (**Figure 3.10.C**). Extrapolation of our findings indicates that the anti-parasitic molecules described by Douch and colleagues are unlikely to be LTs, Relmβ or Arginase but maybe PLA_2g1B. It would be interesting to determine whether PLA_2g1B is responsible resistance in sheep. For example, is *Pla2g1b* expression and PLA_2 activity elevated in resistant sheep intestinal tissue? Are exsheathed L3 *T. colubriformis* and *H. contortus* larvae sensitive to PLA_2g1B *in vitro*, with respect to larval motility and migration (Rabel et al., 1994) as well as their infectivity in naïve sheep. It would also be of interest to adapt the larval migration inhibitory assay (Rabel et al., 1994) and other anthelmintic screens (Hu et al., 2013), for use with PLA_2g1B-treated *H. polygyrus* larvae. This could shed light on how PLA_2g1B affects the function of *H. polygyrus*.

3.3.2.4 Cooperation between PLA_2g1B and type 2 effector mechanisms is required for functional anti-helminth immunity

Type 2 effector mechanisms are essential in mediating resistance to intestinal helminth infections. In the context of *H. polygyrus* infection, IL-4Rα signalling is essential for resistance (Urban et al., 1991b), promoting alternative activation of macrophages (Anthony et al., 2006), B cell class switching and IgG1 production (Katona et al., 1991, Urban et al., 1991a, McCoy et al., 2008) and goblet cell hyperplasia and Relmβ production (Herbert et al., 2009). Here we demonstrate that PLA_2g1B is essential for resistance to *H. polygyrus* and *N. brasiliensis*, with *Pla2g1b*$^{-/-}$ mice unable to clear a 2^{o} *H. polygyrus* challenge infection despite a sizable type 2 immune response (section **3.2.3** and **3.2.4**). Furthermore, we demonstrated that *Pla2g1b* expression was distinct from IL-4 *in vivo* (**Figure 3.20.A**), suggesting that it

may not be regulated by IL-4Rα signalling. Resistance to *H. polygyrus* infection requires the cooperation of type 2 immune responses and the direct anthelmintic effect of PLA_2g1B, with mice deficient in either one failing to eliminate 2^o challenge infection (**Figure 3.14**). Moreover, we show that PLA_2g1B is able to reduce worm burden during a 1^o infection (**Figure 3.12**), overcoming the necessity of a memory type 2 response.

3.3.2.5 Does pancreas-derived PLA_2g1B play a role?

We identified *Pla2g1b* to be expressed in the small intestine, specifically in epithelial cells, and upregulated in resistant mice (section **3.2.1**, **3.2.9** and **3.2.10**). However, *Pla2g1b* is expressed in a variety of tissues in C57BL/6 mice, with expression highest in the glandular stomach, pancreas and small intestine respectively (Eerola et al., 2006). With the majority of research on PLA_2g1B focussing on its role in dietary and biliary lipid digestion following secretion from the pancreas, we observed that pancreas-specific *Pla2g1b* expression levels were unchanged during the *H. polygyrus* 2^o challenge infection model (**Figure 3.18.A**). These data suggests that the protective effects of PLA_2g1B are likely to be dependent upon de novo small intestine-derived *Pla2g1b* expression in resistant mice, we cannot however exclude a role for pancreatic PLA_2g1B.

To test this, we would require a mouse where *Pla2g1b* is flanked by two lox-p sites and a pancreas –specific Cre-recombinase driver. Using the pancreas wide Cre-recombinase *Pdx1*^{Cre} would not be appropriate as it also induces recombination in the duodenum (Magnuson and Osipovich, 2013). Specific deletion of *Pla2g1b* in pancreatic acinar cells using the inducible Cre-recombinase driver *Cela1*$^{Cre\text{-}ER}$ would be more appropriate as *Pla2g1b* expression is restricted to acinar cells in the pancreas (Richmond and Hui, 2000), induces close to 100% activity in acini after tamoxifen treatment (Ji et al., 2008) and does not affect the small intestine (Magnuson and Osipovich, 2013). In addition, to demonstrate that small intestine epithelial cell-derived *Pla2g1b* is essential for resistance to *H. polygyrus,* restricting *Pla2g1b* deficiency to intestinal epithelial cells using the *villin*^{Cre} would not be appropriate as this Cre-recombinase driver also marks acinar cells (Magnuson and

Osipovich, 2013). It would therefore be of benefit to identify the epithelial sub-type that expresses *Pla2g1b* and subsequently use a Cre-recombinase driver specific to that sub-type.

In addition to a genetic approach to restrict pancreatic PLA_2g1B, bile duct ligation would prevent pancreas-derived PLA_2g1B from entering the small intestine. Bile duct ligation in rodents is commonly used to study obstructive cholestatic injury, inducing a strong fibrotic response after 21 days (Tag et al., 2015). This model would allow bile duct ligation prior to 2^o challenge infection with *H. polygyrus* and analysis of luminal worm burden 14 days-post infection. There is a possibility that the fibrotic response would affect antihelminth immunity, therefore potentially confounding the results.

Regardless, if physiological expression of pancreatic *Pla2g1b* contributes to resistance to *H. polygyrus*, it would be of interest to see if super-physiological levels of pancreatic PLA_2g1B can induce resistance upon 1^o infection of *H. polygyrus*. To test this, we could utilise the mouse which over expresses human *PLA2G1B* in pancreatic acinar cells (Cash et al., 2011), and determine luminal *H. polygyrus* load in the small intestine following 1^o infection.

3.3.3 Regulation of *Pla2g1b* expression

3.3.3.1 Microbiota in resistant mice

We identified that upregulation of small intestinal *Pla2g1b in vivo* was abrogated with antibiotic treatment (**Figure3.20**), indicating that changes in the intestinal microbiota is required for *Pla2g1b* upregulation. Intestinal helminth infection has previously been shown to induce changes in the intestinal microbiota (Rausch et al., 2013, Reynolds et al., 2014b, Zaiss et al., 2015, Giacomin et al., 2016, Kannan et al., 2017), however changes in the microbiota following drug-clearance of infection has not been investigated. Following our findings, it would be of great interest to determine the changes in the small intestinal microbiota both susceptible and resistant mice, with and without 2^o infection. To test this, we could use the model used for our tissue RNA sequencing (**Figure 3.1**), but instead harvest both faecal and duodenal samples for

16S rRNA sequencing to determine both the presence and abundance of bacterial species.

Following our observation that small intestinal *Pla2g1b in vivo* was abrogated with antibiotic treatment (**Figure 3.20**), and with PLA_2g1B essential for resistance to *H. polygyrus* (**Figure 3.5**), we demonstrated that antibiotic treatment abrogated resistance and prevented clearance of 2º *H. polygyrus* challenge infection. However, PLA_2g1B treatment of the L3 larvae *in vitro*, prior to 2º infection, failed to rescue protective immunity. This experiment must be repeated to confirm these findings. However, this preliminary data suggests that the abrogation of resistance following antibiotic treatment was not only due to a failure to upregulate *Pla2g1b* expression. We previously demonstrated that for functional killing a clearance of *H. polygyrus* required a functional immune system and direct PLA_2g1B anthelmintic effects (**Figure 3.14**), therefore we can speculate that the antibiotic treatment may have also abrogated the type 2 immune response. Antibiotic treatment can alter both type 2 immune responses and barrier function in the intestine (Stefka et al., 2014). Future studies should address the effect of antibiotic treatment on the immune response following *H. polygyrus* infection in this model. This would establish if antibiotic treatment abrogated protection to *H. polygyrus* due to the failure to both upregulate *Pla2g1b* and mount an effective immune response, or alternatively induce another critical unknown antihelminth mechanism.

In addition to using antibiotic treatment, we could also perform faecal transplant experiments to determine the role of the microbiota in resistant to intestinal helminth infection. Specifically, transferring the faecal content from resistant mice to naïve recipient mice may be sufficient to induce *Pla2g1b* expression and confer resistance. Whether there are differences in the microbiota of individual's resistant to intestinal helminth infection and whether resistance could be induced in susceptible individuals by altering the intestinal microbiota is currently unclear and warrants further study.

3.3.3.2 Requirement of immune cells for *Pla2g1b* expression

As well as the upregulation of *Pla2g1b* expression in resistant mice being dependent upon the microbiota, we also identified that *Rag*$^{-/-}\gamma c^{-/-}$ mice also failed to upregulate

Pla2g1b after clearance of a 1º *H. polygyrus* infection (**Figure 3.20**). This indicates a requirement for *Rag*-dependent adaptive and/or common gamma chain-dependent innate immune cells or signalling in the upregulation of *Pla2g1b* in small intestine epithelial cells. Further experiments are required to identify the specific cell type(s) that are required for *Pla2g1b* upregulation. Initially testing *Rag*$^{-/-}$ and *γc*$^{-/-}$ mice to identify the critical compartment for *Pla2g1b* upregulation in resistant mice, followed by specific innate or adaptive cell knockout mice could provide greater resolution and identification of the critical cell type required for *Pla2g1b* upregulation.

Our data show that both the microbiota and a functional immune system are required for *Pla2g1b* upregulation in epithelial cells of resistant mice. From this we can formulate two hypotheses in how the microbiota and immune cells act together to induce *Pla2g1b* expression.

1. A change in composition of the microbiota maintains or induces a population of immune cells, which in turn, provides a signal to the intestinal epithelial cells to increase *Pla2g1b* expression.
2. The lack of a functional immune compartment in *Rag*$^{-/-}$*γc*$^{-/-}$ mice perturbs the change in microbiota composition, required to provide a signal to the intestinal epithelial cells, which in turn, upregulates *Pla2g1b* expression.

Previous studies have demonstrated that both specific strains of bacteria and bacteria-derived products present in the intestinal tract are essential for the development and maintenance of immune cell populations (Atarashi et al., 2011, Ivanov et al., 2009, Gaboriau-Routhiau et al., 2009, Furusawa et al., 2013). In addition, the intestinal microbiota, both bacteria and protozoa, have been shown to have direct effects on intestinal epithelial cells, shaping gene expression and structure (Howitt et al., 2016, Davison et al., 2017, Quevrain et al., 2016). Furthermore, *Rag1*-deficient mice have been shown to have dysbiosis of intestinal microbiota (Smith et al., 2014). Taken together, this body of literature supports both hypotheses above.

Analysis and comparison of the small intestinal microbiota composition, by 16S sequencing, between mice after drug-clearance of 1º *H. polygyrus* infection and during 1º infection would allow the identification of specific bacterial strains associated with resistance. An increase or decrease in specific bacterial species may

promote *Pla2g1b* expression in resistant mice. In order to determine which hypothesis above is correct, comparison of the resistance-induced changes (drug-clearance of 1° *H. polygyrus* infection) in the small intestinal microbiota between WT and immune-deficient mice, such as *Rag*$^{-/-}$*γc*$^{-/-}$ mice, would be required. If the same changes in microbiota were seen, hypothesis 1 is likely to be correct as *Pla2g1b* is not upregulated in *Rag*$^{-/-}$*γc*$^{-/-}$ mice, despite the same changes in microbiota composition occurring. However, if there were differences in the changes in bacterial species, hypothesis 2 is likely to be correct as the lack of a functional immune system would have prevented changes in the microbiota which are required for *Pla2g1b* upregulation. Additionally, measurement of *Pla2g1b* expression in the small intestine following faecal transfer from WT drug-cured mice, or transfer of the bacterial species or product critical for *Pla2g1b* upregulation, into both naïve WT and *Rag*$^{-/-}$*γc*$^{-/-}$ mice would differentiate between hypothesis 1 and 2 above. If faecal transfer of from WT drug-cured mice to *Rag*$^{-/-}$*γc*$^{-/-}$ mice induced *Pla2g1b* expression in the small intestine, hypothesis 2 is likely to be correct. If faecal transfer does not induce *Pla2g1b* expression in the small intestine of *Rag*$^{-/-}$*γc*$^{-/-}$ mice, hypothesis 1 is likely to be correct.

3.3.3.3 Type 2 immunity and PLA$_2$g1B paradox: IL-4Rα signalling

IL-4Rα is essential for resistance to *H. polygyrus* infection (Urban et al., 1995, Urban et al., 1991b), inducing Th2 differentiation (Urban et al., 1991a, Urban et al., 1991b) and downstream type 2 effector mechanisms (Katona et al., 1991, McCoy et al., 2008, Herbert et al., 2009, Anthony et al., 2006). However, we show that *Pla2g1b* expression, which is essential for resistance to *H. polygyrus* (**Figure 3.5**), is unaltered in resistant mice following IL-4 blockade *in vivo* (**Figure 3.20.A**). These data indicate that *Pla2g1b* expression is not regulated by IL-4. From our *in vivo* experiments, we cannot determine if IL-4Rα signalling regulates *Pla2g1b* expression as IL-13, which is equally or more important in mediating type 2 effector responses (Zhu et al., 1999, Li et al., 1999, Finkelman et al., 1999), is capable of inducing IL-4Rα signalling (Mueller et al., 2002). For this we would have to block both IL-4 and IL-13 simultaneously or, cross the *Il4ra*$^{fl/fl}$ (Jenkins et al., 2013) and *Villin*^{Cre} mice to restrict *Il4ra*-deficency to intestinal epithelial cells.

Interestingly, when we investigated the role of IL-4Rα signalling *in vitro* using intestinal epithelial organoid cultures, we identified that following IL-4 and IL-13 stimulation *Pla2g1b* expression was decreased (**Figure 3.21.A**). This suggests that *Pla2g1b* is negatively regulated by IL-4Rα signalling, contrasting to the *in vivo* data where blocking IL-4 had no effect on *Pla2g1b* expression. However, there may be technical limitations which confound the *in* vitro organoid data. The intestinal organoids were stimulated for 48 hours prior to RNA extraction and gene expression analysis. We know this time period allows for the differentiation and hyperplasia of both goblet and tuft cells (Gerbe et al., 2016). With *Pla2g1b* unlikely to be restricted to tuft or goblet cells (**Figure 3.21.B**), the decrease in *Pla2g1b* expression seen in intestinal organoids upon IL-4 and IL-13 stimulation is likely to be due to a lower abundance of the sub-type of *Pla2g1b* expressing epithelial cells due to the increased abundance and outgrowth of both tuft and goblet cells. To overcome this, we can reduce the stimulation duration of IL-4 and IL-13, analysing gene expression before the expansion of tuft and goblet cells. Alternatively, we could promote the differentiation of the intestinal organoids to different epithelial sub-types (Basak et al., 2017) and compare *Pla2g1b* expression or stimulate each subtype with IL-4 and IL-13, as well as other cytokines, and analyse *Pla2g1b* expression.

3.3.3.4 *Pla2g1b* expression in resistant strains

With PLA_2g1B essential for resistance to *H. polygyrus* (**Figure 3.5**) and different strains of mice known to have differing levels of susceptibility to *H. polygyrus* infection (Reynolds et al., 2012), it would be interesting to identify if *Pla2g1b* expression correlated with resistance to *H. polygyrus* infection in different laboratory mouse strains. Other genetic factors, such as major histocompatibility complex H-2 loci (Behnke and Wahid, 1991) and IL-9 (Behnke et al., 2003), have been associated with resistance to *H. polygyrus*, however *Pla2g1b* has not been directly associated with genetic resistance. Intriguingly, C57BL/6 mice have a genetic mutation in *Pla2g2a,* another $sPLA_2$, which prevents the production of functional PLA_2g2A, unlike the more resistant BALB/c strain. PLA_2g2A is expressed in the intestine of BALB/c mice (Eerola et al., 2006) and is implicated in antimicrobial defence (Okita et al., 2015, Harwig et al., 1995, Murakami et al., 2014). With all $sPLA_2$ enzymes catalysing

the same reaction, but with differing substrate specificities (Nevalainen et al., 2008, Koduri et al., 2002), would other $sPLA_2$ promote resistance to *H. polygyrus* infection or are $sPLA_2$ anthelmintic properties restricted to *Pla2g1b*? And could C57BL/6 mice be more susceptible to 1º *H. polygyrus* infection due to a lack of functional PLA_2g2A?

It is unlikely that other $sPLA_2$ enzymes, expressed in the small intestine, are required for resistance to *H. polygyrus* infection as their expression is not differentially expressed 2º *H. polygyrus* infection (**Figure 3.3.E**), nor is their expression altered in *Pla2g1b*$^{-/-}$ mice upon 2º *H. polygyrus* infection (**Figure 3.11.F**), although PLA_2g2A cannot be ruled out. To test the role of PLA_2g1B and PLA_2g2A in immunity to *H. polygyrus* we would have to backcross the *Pla2g1b*$^{-/-}$ mice to a BALB/c background and compare the luminal worm burden to *Pla2g2a*$^{-/-}$ mice upon 2º *H. polygyrus* infection.

3.3.4 Implications for therapeutics

With *Pla2g1b* shown to be essential in mice for immunity to small intestine-dwelling helminths (**Figure 3.5**), the question arises as to whether the same gene is essential from resistance to small intestine-dwelling helminth infection in humans and livestock.

Initially, it would be interesting to test whether human PLA_2g1B has any impact on human intestinal nematodes, similar to our *H. polygyrus* L3 *in vitro* treatment system (**Figure 3.14.A**). In addition, utilisation of the *in vitro* assays developed to screen anthelmintic compounds against L3 larvae of various helminth species could identify if PLA_2g1B had a role in antihelminth immunity in other mammals, discussed previously in section **3.3.2.2**.

Several immunological parameters correlate with human resistance to intestinal helminth infection, such as IgE or IL-5 (van den Biggelaar et al., 2002, Ndhlovu et al., 1996, Hagan et al., 1991). It would be of interest to analyse *PLA2G1B* expression in small intestinal biopsies from individuals and identify if there is any correlation with resistance to intestinal helminth infection.

Importantly, identifying the precise mechanism of PLA_2g1B's effect on intestinal helminths and the role of parasite-phospholipids in helminth infection and health is

critical, as discussed in section **3.3.2.3**. Identifying these mechanisms could lead to the design of synthetic helminth-specific molecules, leading to the generation of new anthelmintics. Whether this would culminate in another prophylactic treatment or whether this could provide long lasting immunity is unclear.

To provide long lasting immunity to helminth infections vaccines are necessary but, despite progression in mouse models (Hewitson et al., 2011), generation of human vaccines have stalled. With our finding that protective type 2 immune effector mechanisms require PLA_2g1B-mediated anthelmintic effects (**Figure 3.14**), promoting *PLA2G1B* expression may boost vaccine-induced protection to intestinal helminth infection. As mentioned, for this to be implemented, the mechanism of *Pla2g1b* regulation in epithelial cells would need to be precisely determined (discussed in section **3.3.3**) and used to create a therapeutic which can artificially boost and maintain its expression.

3.4 Figures

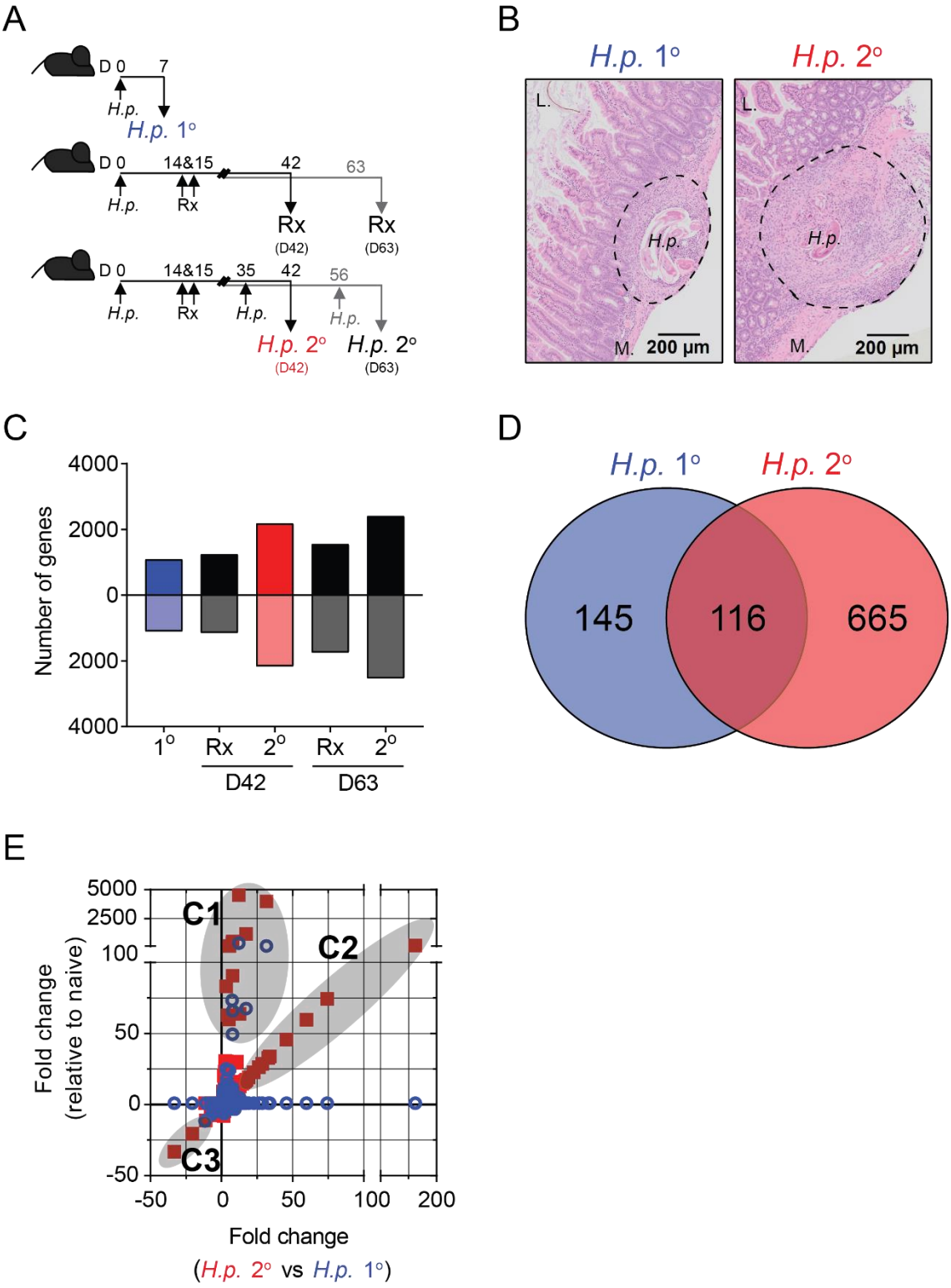

A
D 0
7
H.p.
H.p. 1°
D 0
14&15
42
63
H.p.
Rx
Rx
(D42)
Rx
(D63)
D 0
14&15
35
42
56
H.p.
Rx
H.p.
H.p.
H.p. 2°
(D42)
H.p. 2°
(D63)
B
H.p. 1°
H.p. 2°
L.
H.p.
M.
200 µm
L.
H.p.
M.
200 µm
C
Number of genes
4000
2000
0
2000
4000
1°
Rx
2°
Rx
2°
D42
D63
D
H.p. 1°
H.p. 2°
145
116
665
E
Fold change
(relative to naive)
5000
2500
100
50
0
-50
C1
C2
C3
-50
0
50
100
200
Fold change
(H.p. 2° vs H.p. 1°)

Figure 3.1 RNA sequencing of intestinal tissue reveals distinct gene clusters associated with resistance to *H. polygyrus*

(**A**) C57BL/6 mice were orally infected with 200 L3 *H. polygyrus* larvae on day 0. A cohort of mice was sacrificed 7 days-post 1º *H. polygyrus* infection (*H.p.* 1º). Remaining mice were drug treated (Rx) on days 14 and 15. Two cohorts of mice were then harvest on day 42 or Day 63 (Rx (D42) and Rx (D63), respectively). Another two cohorts of mice were then 2º challenge infected with *H. polygyrus* on day 35 or day 56 and harvested 7 days-post 2º infection (*H.p.* 2º (D42) and *H.p.* 2º (D63), respectively). RNA was harvested from the duodenum at each time point, n=8. (**B**) H&E staining of the small intestine from *H.p.* 1º and *H.p.* 2º (D42). (**C**) The number of genes significantly differentially expressed in *H.p.* 1º, Rx (D42), Rx (D63), *H.p.*2º (D42) and *H.p.*2º (D63) (up- and downregulated, relative to naïve, $p<0.05$). (**D**) Common and differentially expressed genes in *H.p.* 1º and *H.p.* 2º (D42) (relative to naïve, 2-fold filter, $p<0.05$). (**E**) Ratio of ratios analysis of differentially expressed genes in *H.p.* 1º and *H.p.* 2º (D42) (relative to naive, 2-fold filter, $p<0.05$) identified distinct gene clusters (C1-3). See also **Table 3.1**, **Table 3.2** and **Table 3.3** for genes in C1-3.

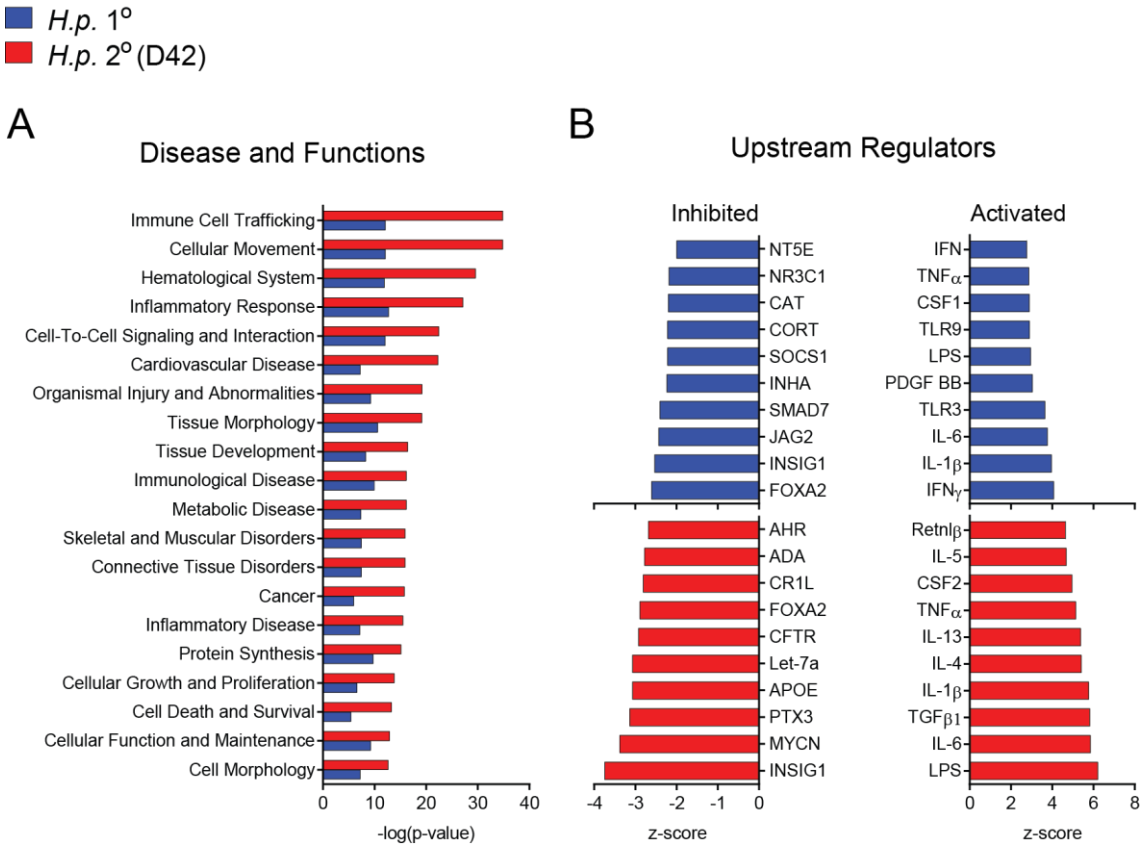


Figure 3.2 Pathway analysis of susceptible and resistant mice

(**A**) Top 20 pathways predicted to be activated in *H.p.* 2° (D42) and *H.p.* 1° (relative to naïve, 2-fold filter, $p<0.05$). (**B**) Top 10 predicated activated and inhibited upstream regulators of *H.p.* 1° and *H.p.* 2° (D42) transcriptomes (relative to naïve, 2-fold filter, $p<0.05$).

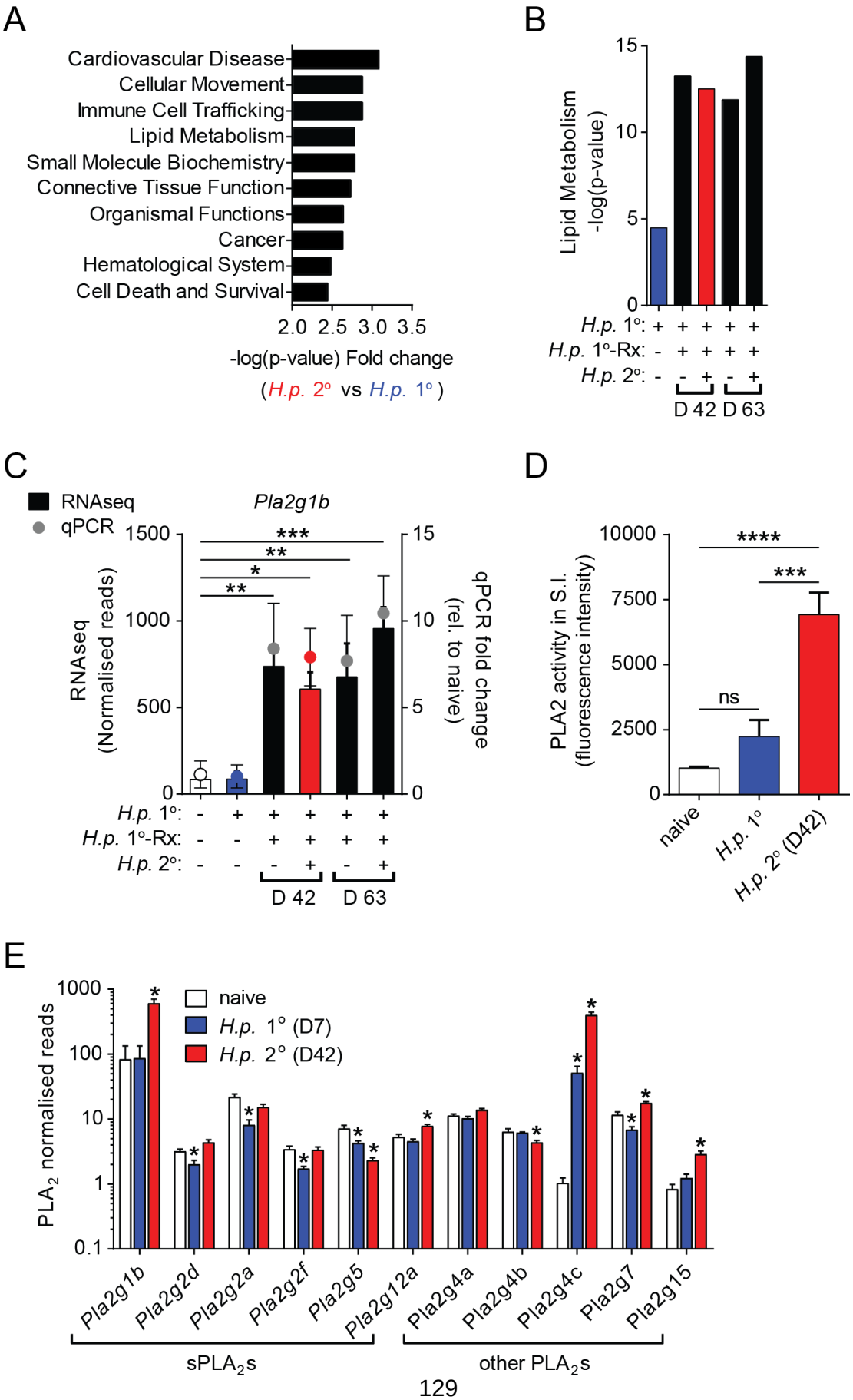

A
Cardiovascular Disease
Cellular Movement
Immune Cell Trafficking
Lipid Metabolism
Small Molecule Biochemistry
Connective Tissue Function
Organismal Functions
Cancer
Hematological System
Cell Death and Survival
2.0 2.5 3.0 3.5
-log(p-value) Fold change
(H.p. 2° vs H.p. 1°)
B
Lipid Metabolism
-log(p-value)
15
10
5
0
H.p. 1°: + + + + +
H.p. 1°-Rx: - + + + +
H.p. 2°: - - + - +
D 42 D 63
C
RNAseq
qPCR
Pla2g1b
RNAseq
(Normalised reads)
qPCR fold change
(rel. to naive)
1500
1000
500
0
15
10
5
0
***
**
*
**
H.p. 1°: - + + + + +
H.p. 1°-Rx: - - + + + +
H.p. 2°: - - - + - +
D 42 D 63
D
PLA2 activity in S.I.
(fluorescence intensity)
10000
7500
5000
2500
0
****
***
ns
naive
H.p. 1°
H.p. 2° (D42)
E
PLA2 normalised reads
1000
100
10
1
0.1
naive
H.p. 1° (D7)
H.p. 2° (D42)
Pla2g1b
Pla2g2d
Pla2g2a
Pla2g2f
Pla2g5
Pla2g12a
Pla2g4a
Pla2g4b
Pla2g4c
Pla2g7
Pla2g15
sPLA2s
other PLA2s

Figure 3.3 RNA sequencing and pathway analysis identified *Pla2g1b* and lipid metabolism pathways to be upregulated in resistant mice

(**A**) Top 10 pathways predicted to be activated more highly in and *H.p.* 2º (D42) than *H.p.* 1º (relative to naïve, 2-fold filter, $p<0.05$). (**B**) Lipid metabolism pathway predicted activation score (relative to naïve, 2-fold filter, $p<0.05$). (**C**) *Pla2g1b* expression in small intestine from RNA sequencing data, confirmed by qPCR. (**D**) PLA_2 activity in the small intestine of naïve, *H.p.* 1º and *H.p.* 2º mice. (**E**) Phospholipase A_2 isoform expression in the small intestine from RNA sequencing. Data represented as mean ± SEM, n=6-8. ns = not significant, *** = $p<0.001$, **** = $p<0.0001$ determined using a one-way ANOVA with Dunnett's or Tukey's multiple comparison analysis.

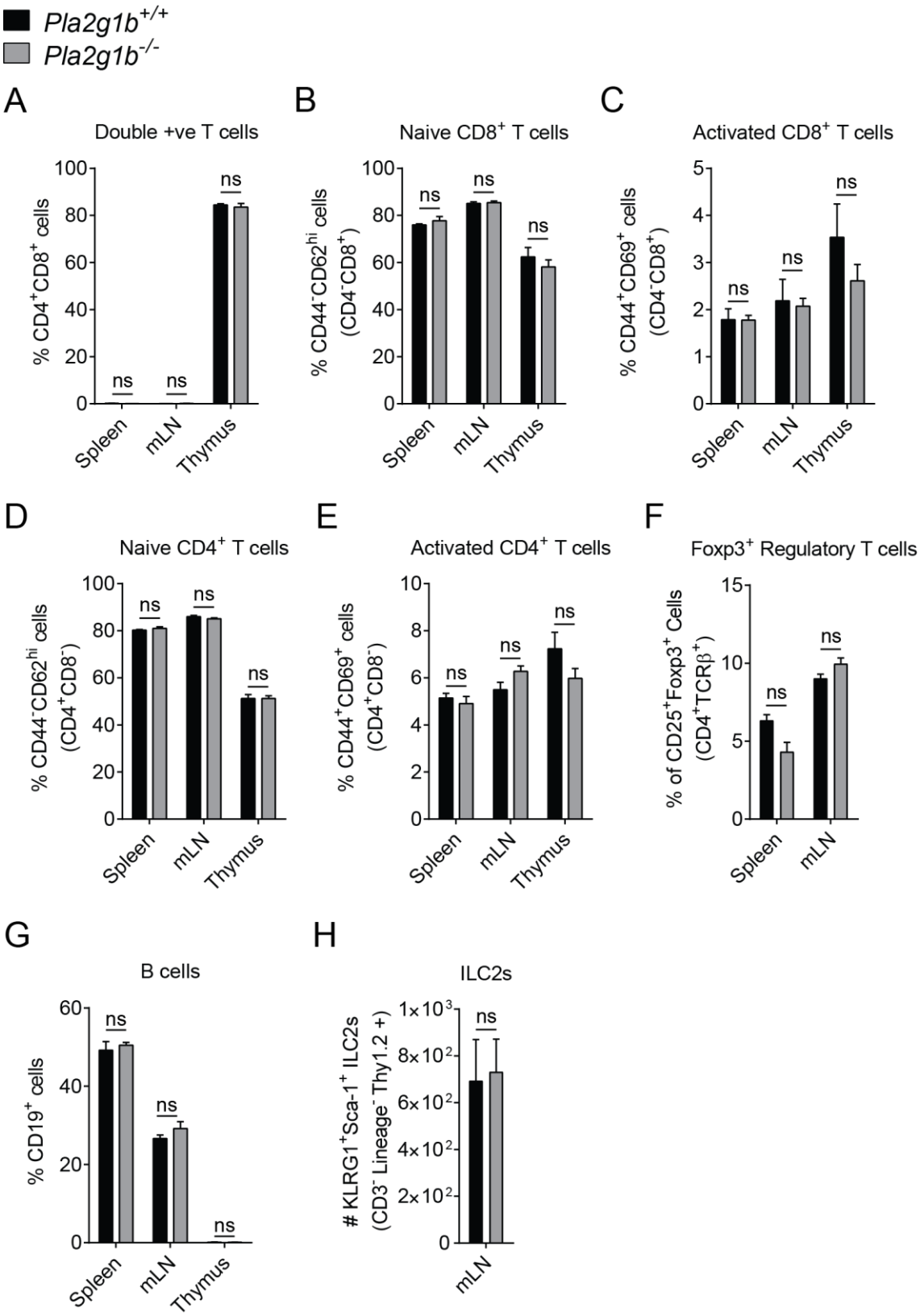

Pla2g1b+/+
Pla2g1b-/-
A
Double +ve T cells
% CD4+CD8+ cells
B
Naive CD8+ T cells
% CD44-CD62hi cells (CD4-CD8+)
C
Activated CD8+ T cells
% CD44+CD69+ cells (CD4-CD8+)
D
Naive CD4+ T cells
% CD44-CD62hi cells (CD4+CD8-)
E
Activated CD4+ T cells
% CD44+CD69+ cells (CD4+CD8-)
F
Foxp3+ Regulatory T cells
% of CD25+Foxp3+ Cells (CD4+TCRβ+)
G
B cells
% CD19+ cells
H
ILC2s
KLRG1+Sca-1+ ILC2s (CD3-Lineage-Thy1.2 +)
ns
Spleen
mLN
Thymus

Figure 3.4 *Pla2g1b*$^{-/-}$ mice do not display any immune defects at baseline

Immune cell frequency was assessed in naïve mice by flow cytometry. (**A**) $CD4^+CD8^+$ T cells. (**B**) Naïve $CD8^+$ T cells. (**C**) Activated $CD8^+$ T cells. (**D**) Naïve $CD4^+$ cells. (**E**) Activated $CD4^+$ cells. (**F**) $Foxp3^+$ Regulatory T cells. (**G**) B cell frequency (**H**) Total number of ILC2s in the mLN. Data represented as mean ± SEM, n=5-6. All data is representative of at least two independent experiments. ns = not significant, determined using an unpaired two-tailed t test.

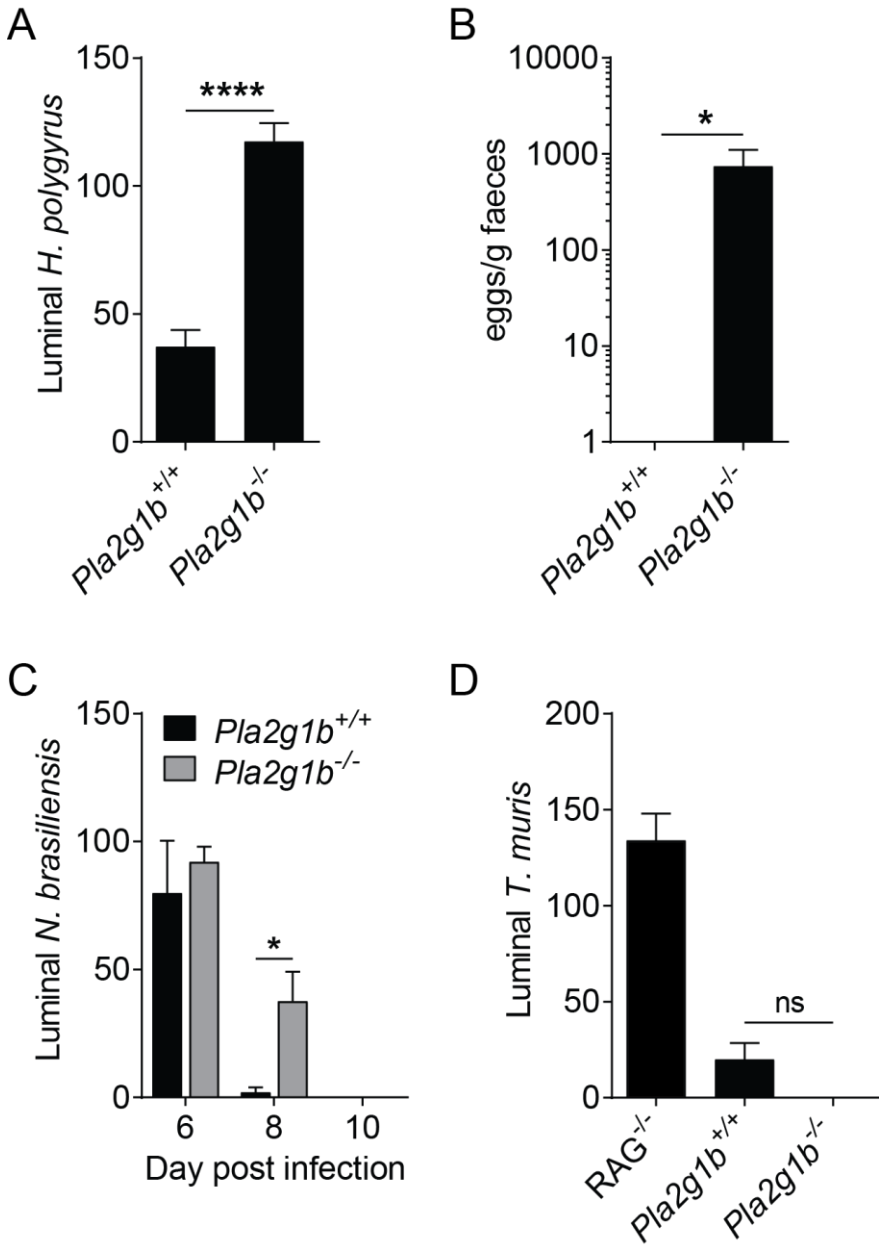


Figure 3.5 *Pla2g1b* is essential for resistance to small intestinal helminths

(**A**) Luminal *H. polygyrus* worms in the small intestine 14 days-post 2º infection. (**B**) Faecal egg burden 14 days-post 2º *H. polygyrus* infection, no eggs were seen in *Pla2g1b*$^{+/+}$ mice. (**C**) Luminal *N. brasiliensis* worms in the small intestine 6, 8 and 10 days-post infection. (**D**) Luminal *T. muris* worms in the cecum and large intestine 35 days-post infection. *Rag*$^{-/-}$ mice were used as an additional control to confirm the infectious dose administered. Data represented as mean ± SEM, n=5-6. All data is representative of at least two independent experiments. ns = not significant, * = p<0.05, **** = p< 0.0001, determined using an unpaired two-tailed t test.

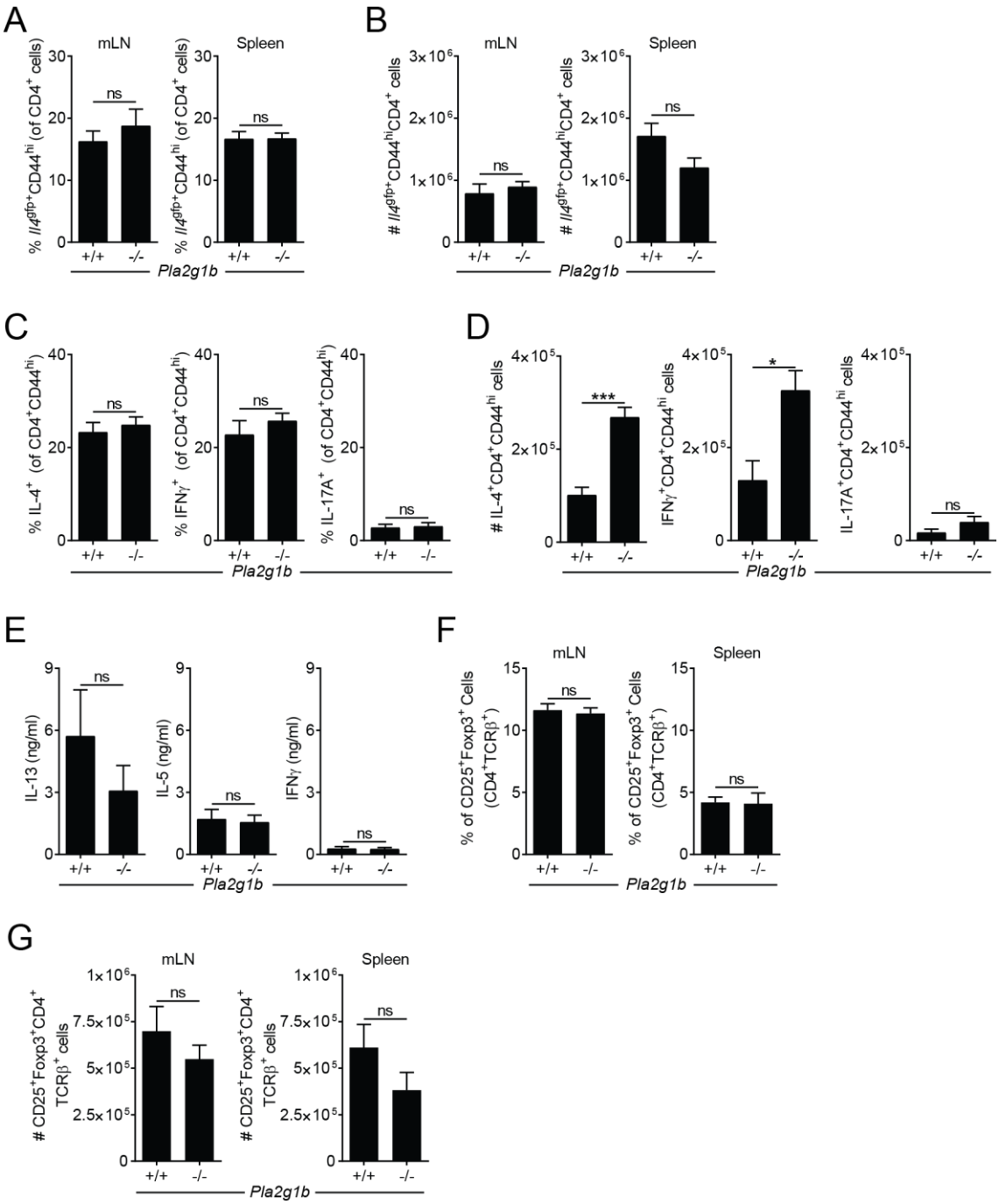

A
mLN
Spleen
% Il4gfp+CD44hi (of CD4+ cells)
ns
+/+
-/-
Pla2g1b
B
mLN
Spleen
Il4gfp+CD44hiCD4+ cells
3x10^6
2x10^6
1x10^6
0
ns
C
% IL-4+ (of CD4+CD44hi)
% IFNγ+ (of CD4+CD44hi)
% IL-17A+ (of CD4+CD44hi)
40
20
0
D
IL-4+CD4+CD44hi cells
IFNγ+CD4+CD44hi cells
IL-17A+CD4+CD44hi cells
4x10^5
2x10^5
***
*
E
IL-13 (ng/ml)
IL-5 (ng/ml)
IFNγ (ng/ml)
9
6
3
0
F
mLN
Spleen
% of CD25+Foxp3+ Cells (CD4+TCRβ+)
15
10
5
G
mLN
Spleen
CD25+Foxp3+CD4+ TCRβ+ cells
1x10^6
7.5x10^5
5x10^5
2.5x10^5

Figure 3.6 T cell response is intact in *Pla2g1b*$^{-/-}$ mice following 2^{o} *H. polygyrus* infection

Frequency (**A**) and total number (**B**) of *Il4*$^{gfp+}$CD44^{hi} CD4^{+} cells in the mesenteric lymph node (mLN) and the spleen 7 days-post 2^{o} infection. Frequency (**C**) and total number (**D**) of cytokine producing T cells in the mLN 7 days-post 2^{o} infection. (**E**) *ex vivo H. polygyrus* antigen-specific cytokine production from the mLN 7 days-post 2^{o} infection. Frequency (**F**) and total number (**G**) of Foxp3^{+} regulatory T cells in the mLN and spleen 7 days-post 2^{o} infection. Data represented as mean ± SEM, n=5-6, representative of at least two independent experiments, ns = not significant, * = p<0.05, *** = p< 0.001 determined using an unpaired two-tailed t test.

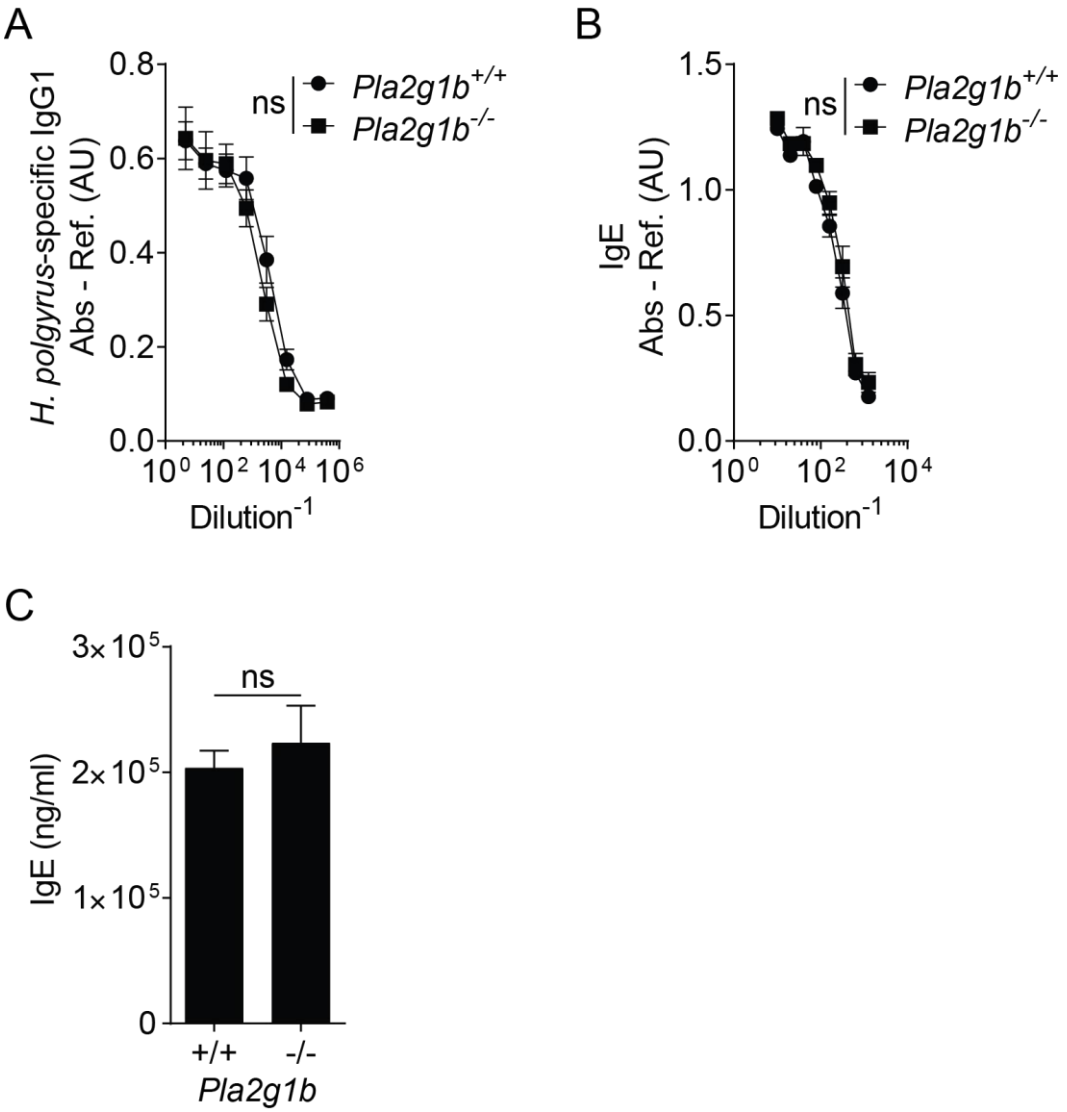


Figure 3.7 Antibody responses are intact in *Pla2g1b*$^{-/-}$ following 2^{o} *H. polygyrus* infection

(**A**) *H.* polygyrus-specific IgG1 in the serum from mice 7 days-post 2^{o} *H.* polygyrus infection (**B**) IgE in the serum from mice 7 days-post 2^{o} *H.* polygyrus infection, quantified in (**C**). Data represented as mean ± SEM, n=5-6. All data is representative of at least two independent experiments. ns = not significant, determined using a two-way ANOVA with Sidak's multiple comparison analysis or an unpaired two-tailed t test.

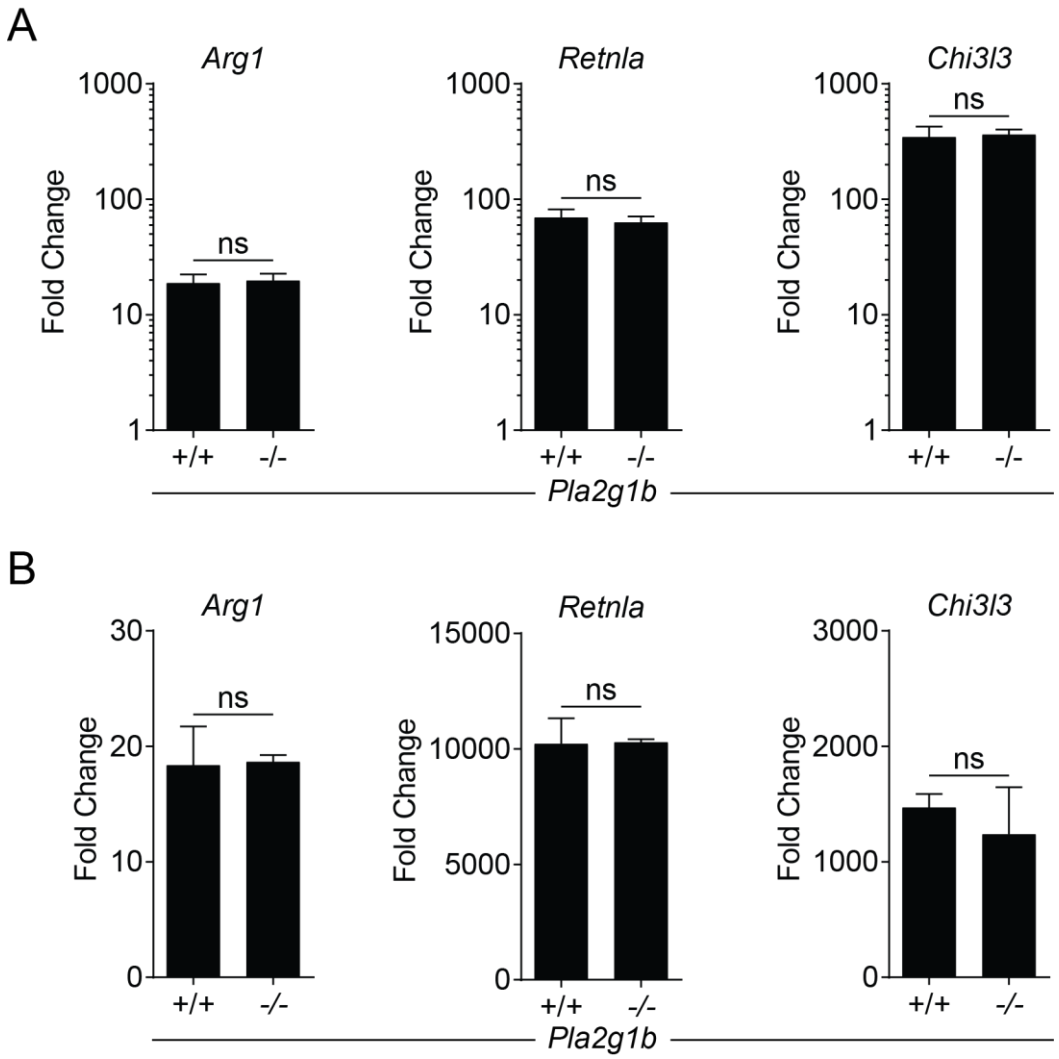


Figure 3.8 *Pla2g1b*-deficiency does not perturb alternate activation of macrophages

(**A**) Alternately activated macrophage marker gene expression in the small intestine of mice 7 days-post 2^{o} *H.* polygyrus infection. (**B**) Bone marrow-derived macrophages were stimulated with IL-4 and IL-13 for 24 hours and *Arg1*, *Retnla* and *Chi3l3* gene expression was analysed. Data represented as mean ± SEM, n=5-6. All data is representative of at least two independent experiments. ns = not significant, determined using an unpaired two-tailed t test.

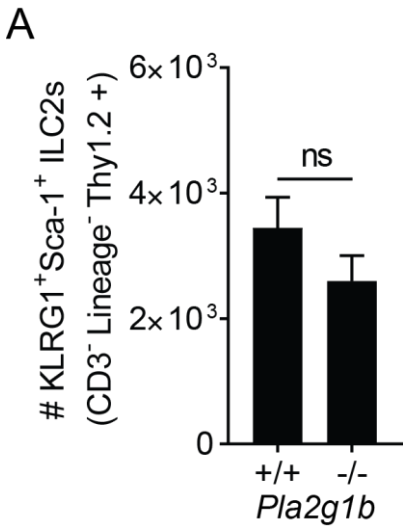


Figure 3.9 *Pla2g1b*-deficency does not alter the ILC2 population following 2º *H. polygyrus* infection

(**A**) Total numbers of ILC2 cells in the mLN 7 days-post 2º *H.* polygyrus infection. Data represented as mean ± SEM, n=5, representative of at least two independent experiments. ns = not significant, determined using an unpaired two-tailed t test.

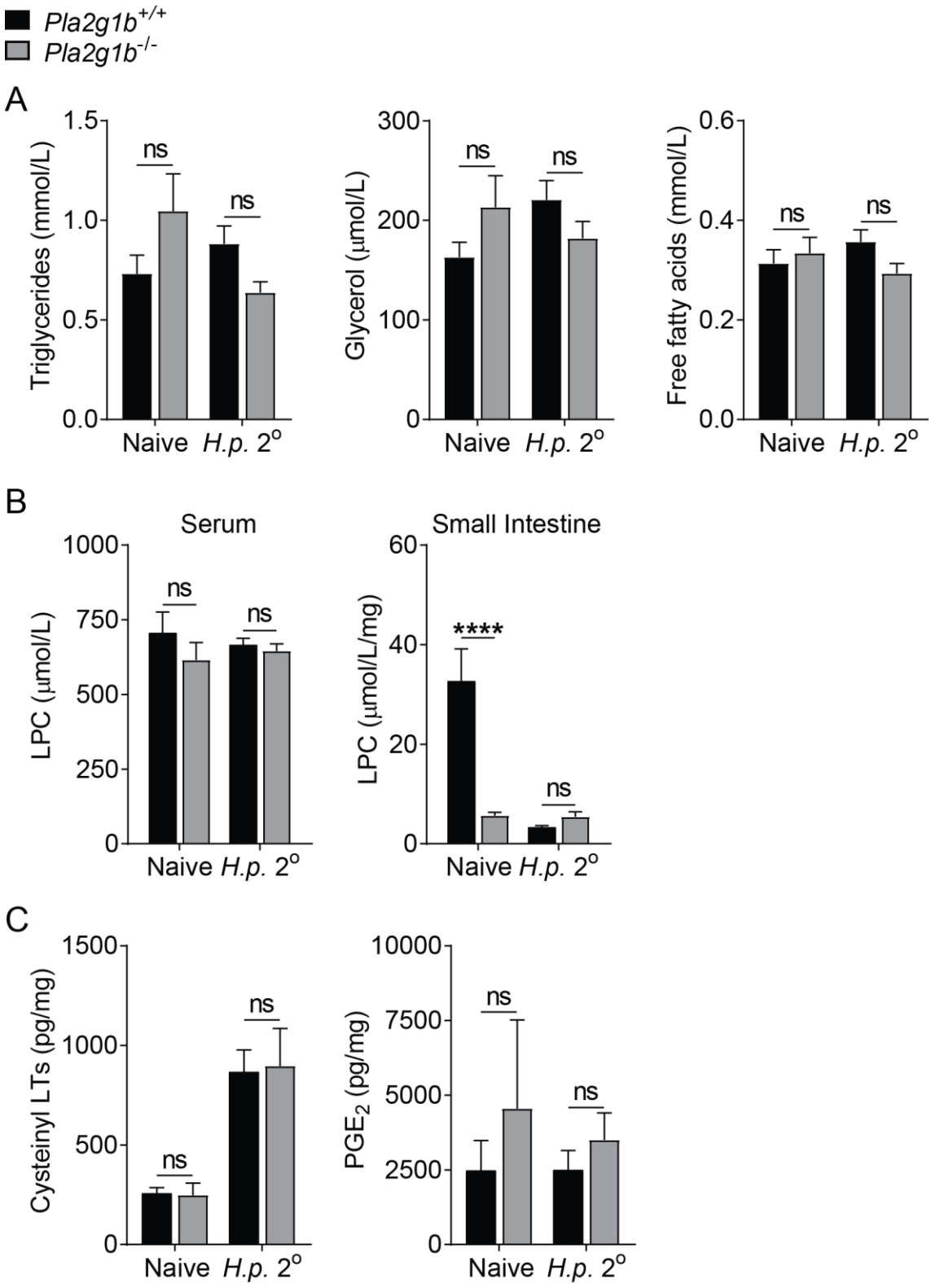

Pla2g1b+/+
Pla2g1b-/-
A
Triglycerides (mmol/L)
1.5
1.0
0.5
0.0
ns
ns
Naive
H.p. 2°
Glycerol (µmol/L)
300
200
100
0
ns
ns
Naive
H.p. 2°
Free fatty acids (mmol/L)
0.6
0.4
0.2
0.0
ns
ns
Naive
H.p. 2°
B
Serum
LPC (µmol/L)
1000
750
500
250
0
ns
ns
Naive
H.p. 2°
Small Intestine
LPC (µmol/L/mg)
60
40
20
0
****
ns
Naive
H.p. 2°
C
Cysteinyl LTs (pg/mg)
1500
1000
500
0
ns
ns
Naive
H.p. 2°
PGE2 (pg/mg)
10000
7500
5000
2500
0
ns
ns
Naive
H.p. 2°

Figure 3.10 Lipid metabolism and bioactive lipid synthesis is intact in *Pla2g1b*-deficient mice

(**A**) Lipid metabolites in the serum of naïve and 7 days-post 2^{o} *H.* polygyrus infected mice. (**B**) Lysophosphatidylcholine (LPC) concentration in the serum and small intestinal homogenate of naïve and 7 days-post 2^{o} *H.* polygyrus infected mice. (**C**) Cysteinyl leukotrienes (LTs) and Prostaglandin E_2 (PGE_2) concentration in the small intestine of naïve and 7 days post 2^{o} *H.* polygyrus infected mice. Data represented as mean ± SEM, n=4-6. All data is representative of at least two independent experiments. ns = not significant, determined using a two-way ANOVA with Sidak's multiple comparison analysis.

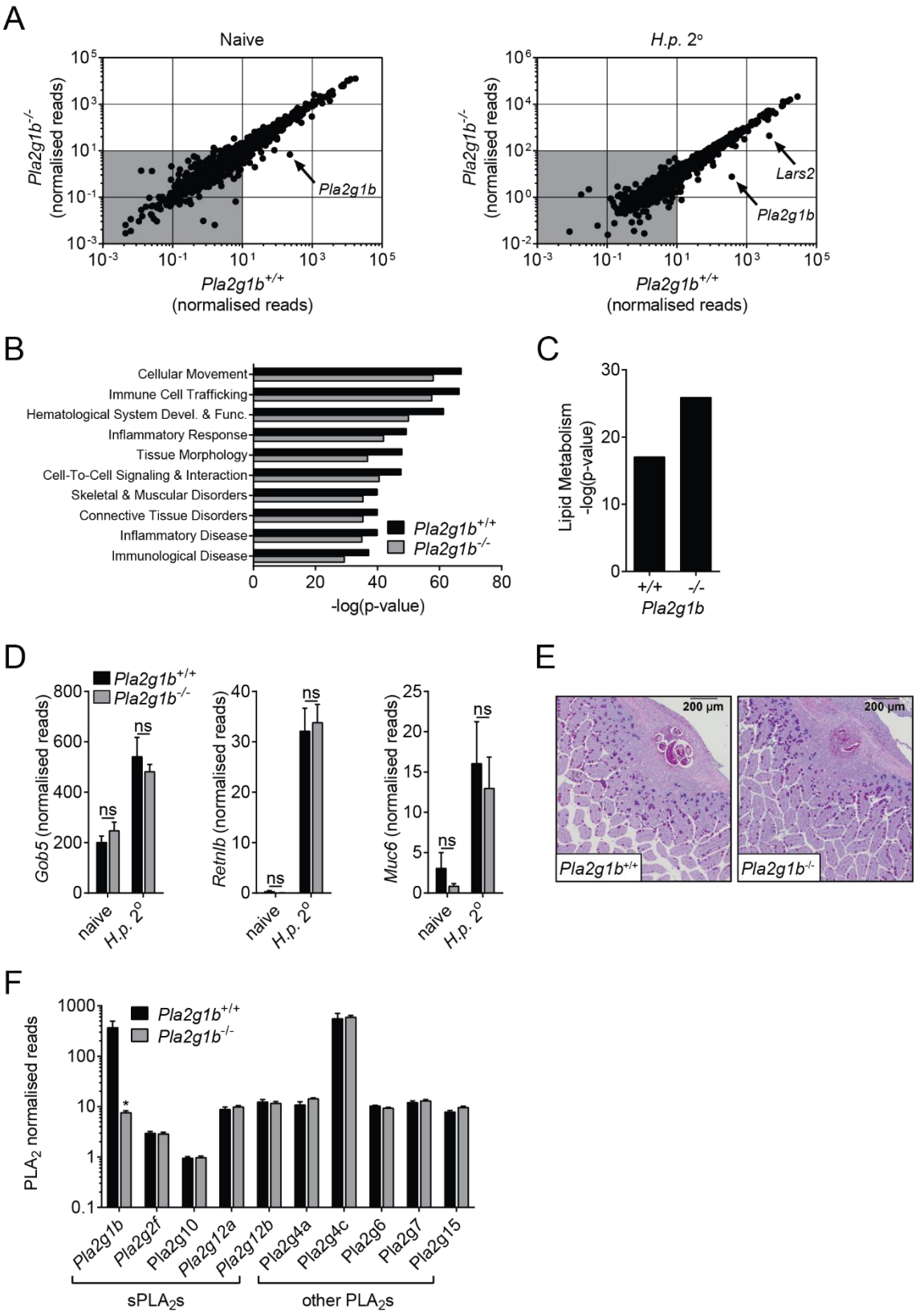

A
Naive
H.p. 2°
Pla2g1b-/- (normalised reads)
Pla2g1b+/+ (normalised reads)
Pla2g1b
Lars2
B
Cellular Movement
Immune Cell Trafficking
Hematological System Devel. & Func.
Inflammatory Response
Tissue Morphology
Cell-To-Cell Signaling & Interaction
Skeletal & Muscular Disorders
Connective Tissue Disorders
Inflammatory Disease
Immunological Disease
Pla2g1b+/+
Pla2g1b-/-
-log(p-value)
C
Lipid Metabolism
-log(p-value)
+/+
-/-
Pla2g1b
D
Gob5 (normalised reads)
Retnlb (normalised reads)
Muc6 (normalised reads)
ns
naive
H.p. 2°
E
200 µm
Pla2g1b+/+
Pla2g1b-/-
F
PLA2 normalised reads
Pla2g1b
Pla2g2f
Pla2g10
Pla2g12a
Pla2g12b
Pla2g4a
Pla2g4c
Pla2g6
Pla2g7
Pla2g15
sPLA2s
other PLA2s

Figure 3.11 RNA sequencing of the small intestine reveals no significant differences in *Pla2g1b*$^{-/-}$ mice

(**A**) RNA sequencing-generated transcriptional landscape of the small intestine of naïve and 7 days-post 2^{o} infection WT (*Pla2g1b*$^{+/+}$) and *Pla2g1b*$^{-/-}$ mice. (**B**) Top 10 predicated pathways 7 days-post 2^{o} *H. polygyrus* infection of WT (*Pla2g1b*$^{+/+}$) and *Pla2g1b*$^{-/-}$ mice (relative to naïve, 2-fold filter, $p<0.05$). (**C**) Lipid metabolism pathway predicted activation score in WT (*Pla2g1b*$^{+/+}$) and *Pla2g1b*$^{-/-}$ mice 7 days-post 2^{o} *H. polygyrus* infection (relative to naïve, 2-fold filter, $p<0.05$). (**D**) Goblet cell-associated gene expression in the small intestine of naïve and 7 days-post 2^{o} *H.* polygyrus infected WT and *Pla2g1b*$^{-/-}$ mice. (**E**) Mucus staining (Alcian blue-periodic acid Schiff) of the small intestine from mice 7 days-post 2^{o} *H.* polygyrus infection. (**F**) Phospholipase A_2 isoform expression in the small intestine 7 days-post 2^{o} *H. polygyrus* infection of WT (*Pla2g1b*$^{+/+}$) and *Pla2g1b*$^{-/-}$ mice. Data represented as mean ± SEM, n=5-6, ns = not significant, * = $p<0.05$, determined in RNA sequencing analysis (see methods).

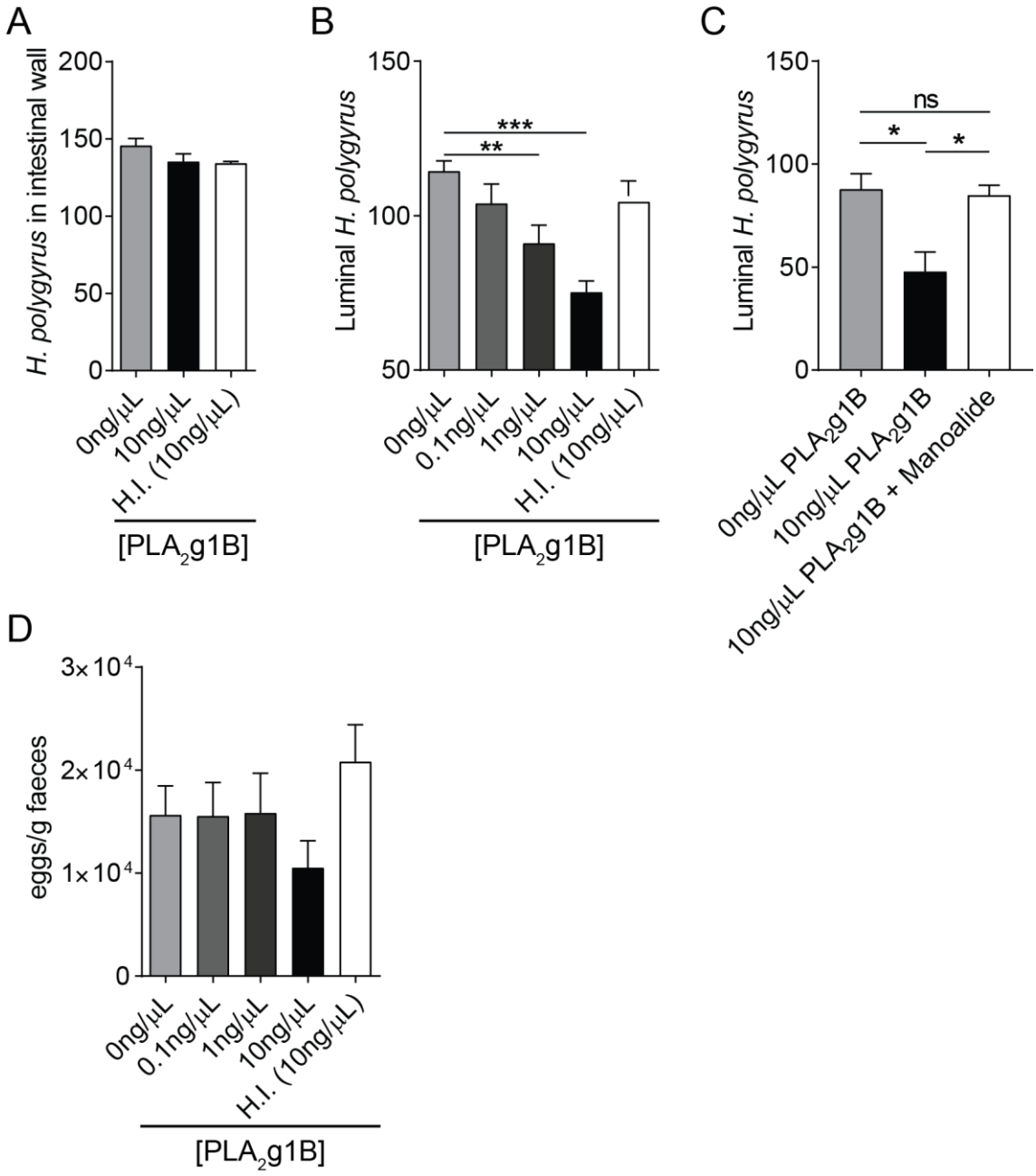

A
H. polygyrus in intestinal wall
200
150
100
50
0
0ng/µL
10ng/µL
H.I. (10ng/µL)
[PLA2g1B]
B
Luminal H. polygyrus
150
100
50
***
**
0ng/µL
0.1ng/µL
1ng/µL
10ng/µL
H.I. (10ng/µL)
[PLA2g1B]
C
Luminal H. polygyrus
150
100
50
0
ns
*
*
0ng/µL PLA2g1B
10ng/µL PLA2g1B
10ng/µL PLA2g1B + Manoalide
D
eggs/g faeces
3×10^4
2×10^4
1×10^4
0
0ng/µL
0.1ng/µL
1ng/µL
10ng/µL
H.I. (10ng/µL)
[PLA2g1B]

Figure 3.12 PLA_2g1B has direct anthelmintic properties which prevent establishment of *H. polygyrus*

(**A**) Number of *H. polygyrus* larvae embedded in the small intestinal wall 5 days-post infection following 24-hour *in vitro* treatment of L3 larvae with PLA_2g1B, n=5. (**B**) Luminal *H. polygyrus* worms in the small intestine 14 days-post 1^o infection following 24-hour *in vitro* treatment of L3 larvae with PLA_2g1B, n=10 (data pooled from two independent experiments). (**C**) Luminal *H. polygyrus* worms in the small intestine 14 days-post 1^o infection following 24-hour *in vitro* treatment of L3 larvae with PLA_2g1B ± Manoalide (200 ng/µL), n=5. (**D**) Faecal egg count 14 days-post 1^o infection following 24-hour *in vitro* treatment of L3 larvae with PLA_2g1B, n=10 (data pooled from two independent experiments). All data is representative of at least two independent experiments. ns = not significant, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$ determined using a one-way ANOVA with Tukey's or Dunnett's multiple comparison analysis.

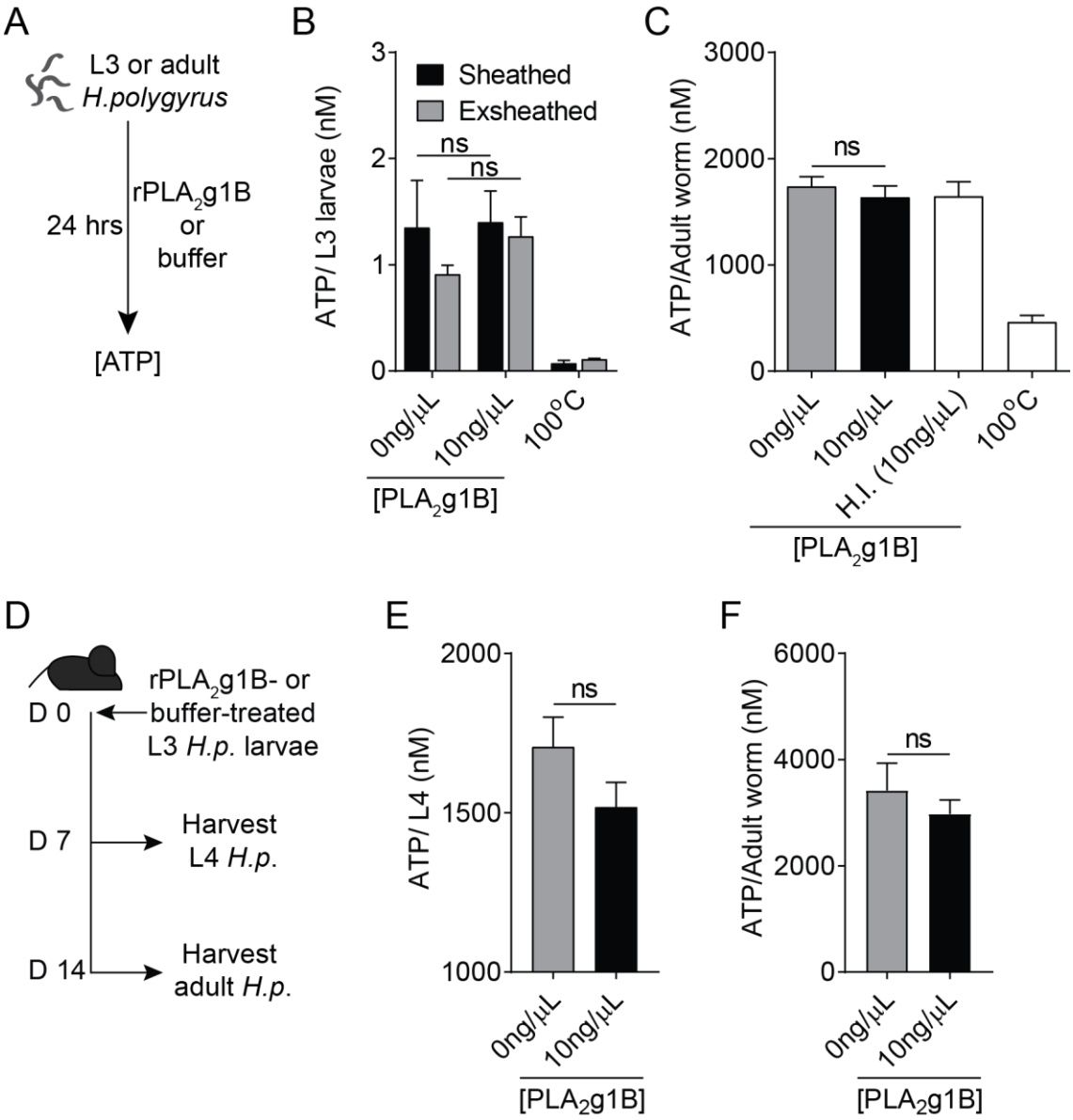

A
L3 or adult
H.polygyrus
24 hrs
rPLA2g1B
or
buffer
[ATP]
B
ATP/ L3 larvae (nM)
Sheathed
Exsheathed
ns
ns
0ng/μL
10ng/μL
100°C
[PLA2g1B]
C
ATP/Adult worm (nM)
ns
0ng/μL
10ng/μL
H.I. (10ng/μL)
100°C
[PLA2g1B]
D
rPLA2g1B- or
buffer-treated
L3 H.p. larvae
D 0
D 7
Harvest
L4 H.p.
D 14
Harvest
adult H.p.
E
ATP/ L4 (nM)
ns
0ng/μL
10ng/μL
[PLA2g1B]
F
ATP/Adult worm (nM)
ns
0ng/μL
10ng/μL
[PLA2g1B]

Figure 3.13 PLA_2g1B treatment does not compromise the fitness of *H. polygyrus*

(**A**) Protocol of *in vitro* PLA_2g1B treatment of *H. polygyrus* L3 larvae and L5 adult worms for 24-hours, followed by ATP concentration analysis. (**B**) ATP concentration of L3 *H. polygyrus* larvae following 24-hour PLA_2g1B treatment. n=3. (**C**) ATP concentration of adult L5 *H. polygyrus* following 24-hour PLA_2g1B treatment. (**D**) Protocol of *ex* vivo ATP analysis of L4 and L5 adult *H.* polygyrus. L3 larvae were treated in *vitro* with 10 ng/µL PLA_2g1B for 24-hours before infection of C57BL/6 mice. Mice were then harvested at 7- and 14 days-post infection to harvest L4 and L5 *H. polygyrus* respectively and ATP concentration was then analysed. (**E**) ATP concentration of L4 *H. polygyrus* isolated from the small intestine 7 days-post infection following 24-hour treatment with PLA_2g1B, n=40 (data pooled from three independent experiments). (**F**) ATP concentration of L5 adult *H. polygyrus* isolated from the small intestine 14 days-post infection following 24-hour treatment with PLA_2g1B, n=12. All data is representative of at least two independent experiments. ns = not significant, determined using a one-way ANOVA with Dunnett's multiple comparison analysis or unpaired two-tailed t test.

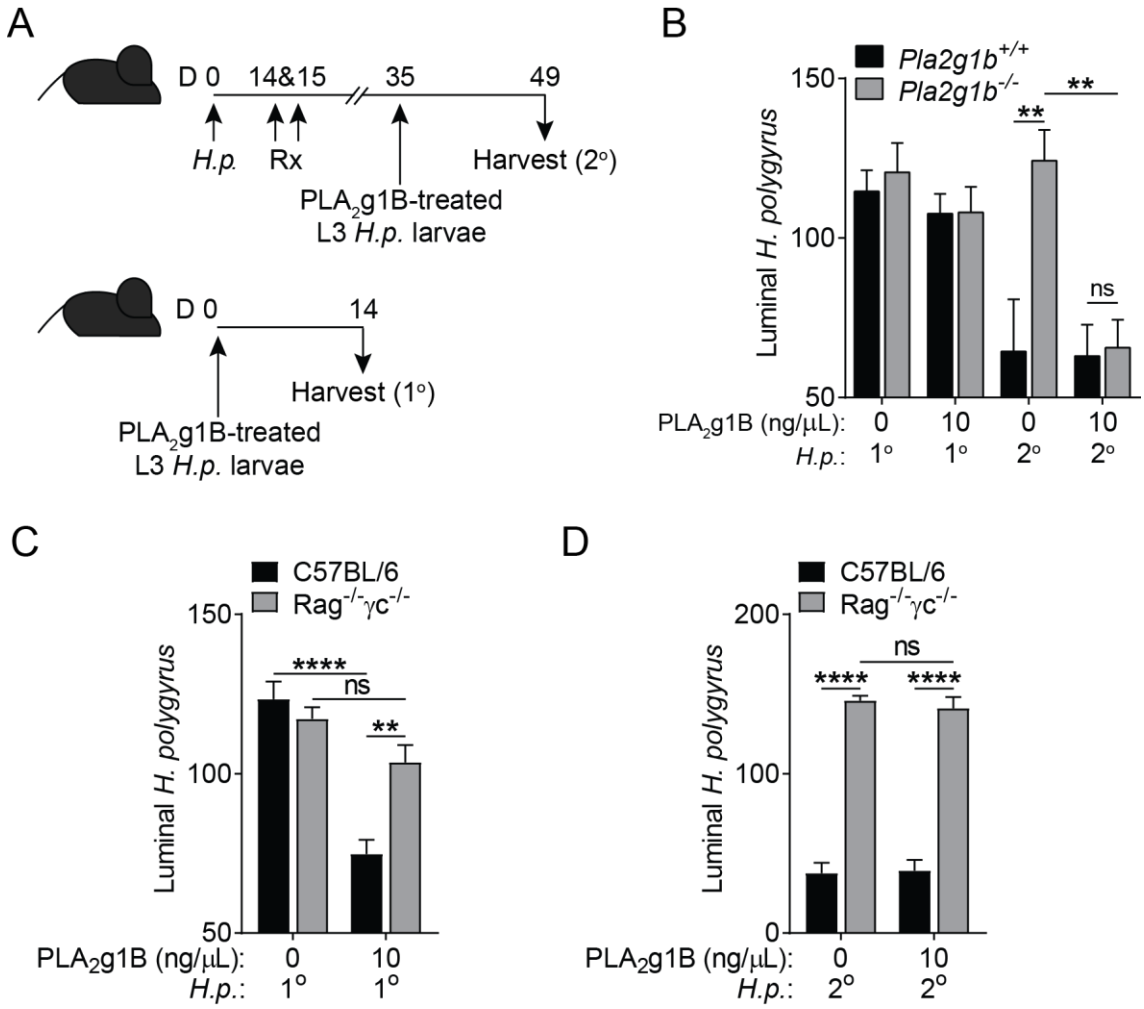


Figure 3.14 PLA_2g1B acts in synergy with type 2 immunity to induce protection against *H. polygyrus* infection

(**A**) Mice were orally infected with 200 L3 *H. polygyrus* (*H.p.*) larvae on day 0 and were drug treated (Rx) on days 14 and 15. Mice were then reinfected with PLA_2g1B-treated L3 *H. p* larvae on day 35 and harvested 14 days-post infection (2°). Another cohort of *Pla2g1b*$^{-/-}$ or WT mice were orally infected with 200 L3 *H. polygyrus* (*H.p.*) larvae on day 0 and harvested 14 days-post infection (1°). (**B**) Luminal *H. polygyrus* worms in the small intestine 14 days-post 1° or 2° infection following 24-hour treatment with PLA_2g1B. (**C**) Luminal *H. polygyrus* worms in the small intestine 14 days-post 1° infection following 24-hour treatment with PLA_2g1B. (**D**) Luminal *H. polygyrus* worms in the small intestine 14 days post 2° infection following 24-hour treatment with PLA_2g1B. Data represented as mean ± SEM, n=4-5. All data is representative of at least two independent experiments, ns = not significant, ** = p<0.01, **** = p< 0.0001 determined using a two-way ANOVA with Sidak's multiple comparison analysis.

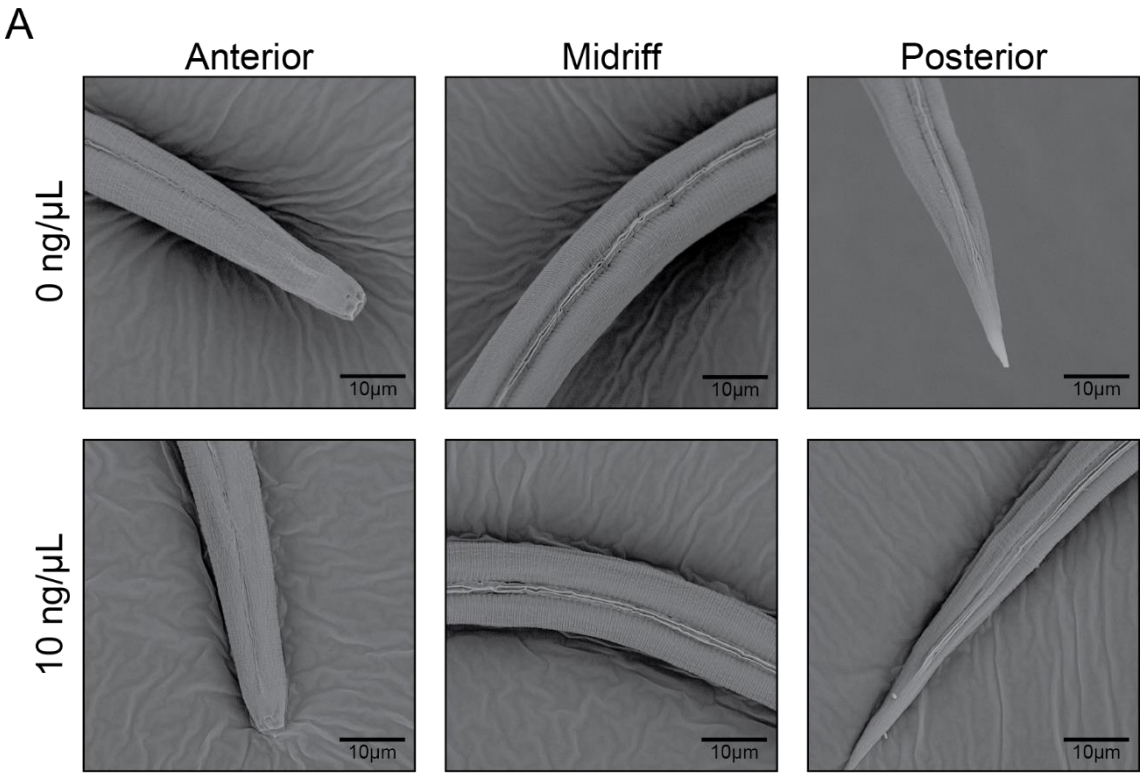


Figure 3.15 Scanning electron microscopy reveals no overt structural changes in PLA_2g1B-treated L3 *H. polygyrus* larvae

(**A**) Scanning electron microscopy of vehicle- or PLA_2g1B-treated exsheathed L3 *H. polygyrus* larvae.

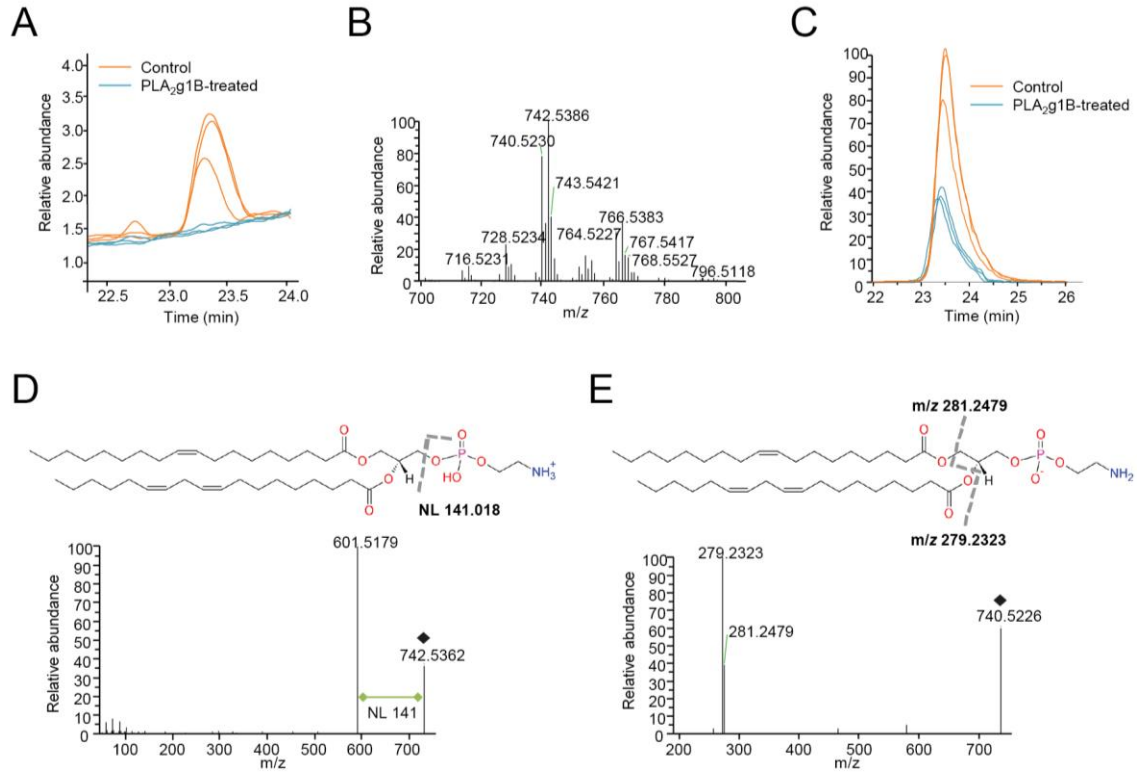


Figure 3.16 LC-MS/MS identifies that PLA_2g1B treatment decreases phosphatidylethanolamine (PE) abundance in L3 *H. polygyrus* larvae

(**A**) Relevant section of the base peak chromatogram of control and PLA_2g1B-treated larvae showing elution of PE phospholipids in negative ion mode. (**B**) Example spectrum from the section of the chromatogram shown in (**A**) at the approximate retention time of PE species, 23.23-23.40 minutes. PEs consist of a phosphoethanolamine head group attached to a glycerol backbone, itself attached to two fatty acid moieties via phosphoether and ester bonds, respectively. As a result, PE MS spectra display traits of fatty acid-containing molecules (clusters that have inter-cluster mass shifts of 28 Da (CH_2CH_2) and intra-cluster mass shifts of 2 Da (indicative of difference in double bond number (fatty acid saturation)). The six identified and three putatively identified PEs are shown in **Figure 3.17.A-B**. (**C**) Extracted ion chromatogram of the ion corresponding to PE 36:3 (18:1, 18:2) in negative ion mode (m/*z* 740.5226). (**D**) Positive-ion MS/MS was used to confirm assignment of the peak as PE, where neutral loss of 141 Da indicates phopshoethanolamine head group. Here, we show the fragmentation of the ion corresponding to PE 36:3 (18:1, 18:2) in positive ion mode (m/*z* 742.5362). (**E**) Negative ion fragmentation of the ion corresponding to PE 36:3 (18:1, 18:2) (m/*z* 740.5226). By identifying both fatty moieties, the individual molecular species were recognized (in this instance, 18:1 and 18:2). Both, the arrangement of the fatty acid moieties at the glycerol backbone (i.e. *sn*-1 or *sn*-2) and the position of the double bonds could not be inferred. LC-MS/MS experiments were performed and analysed by Mariana Silva dos Santos and James MacRae.

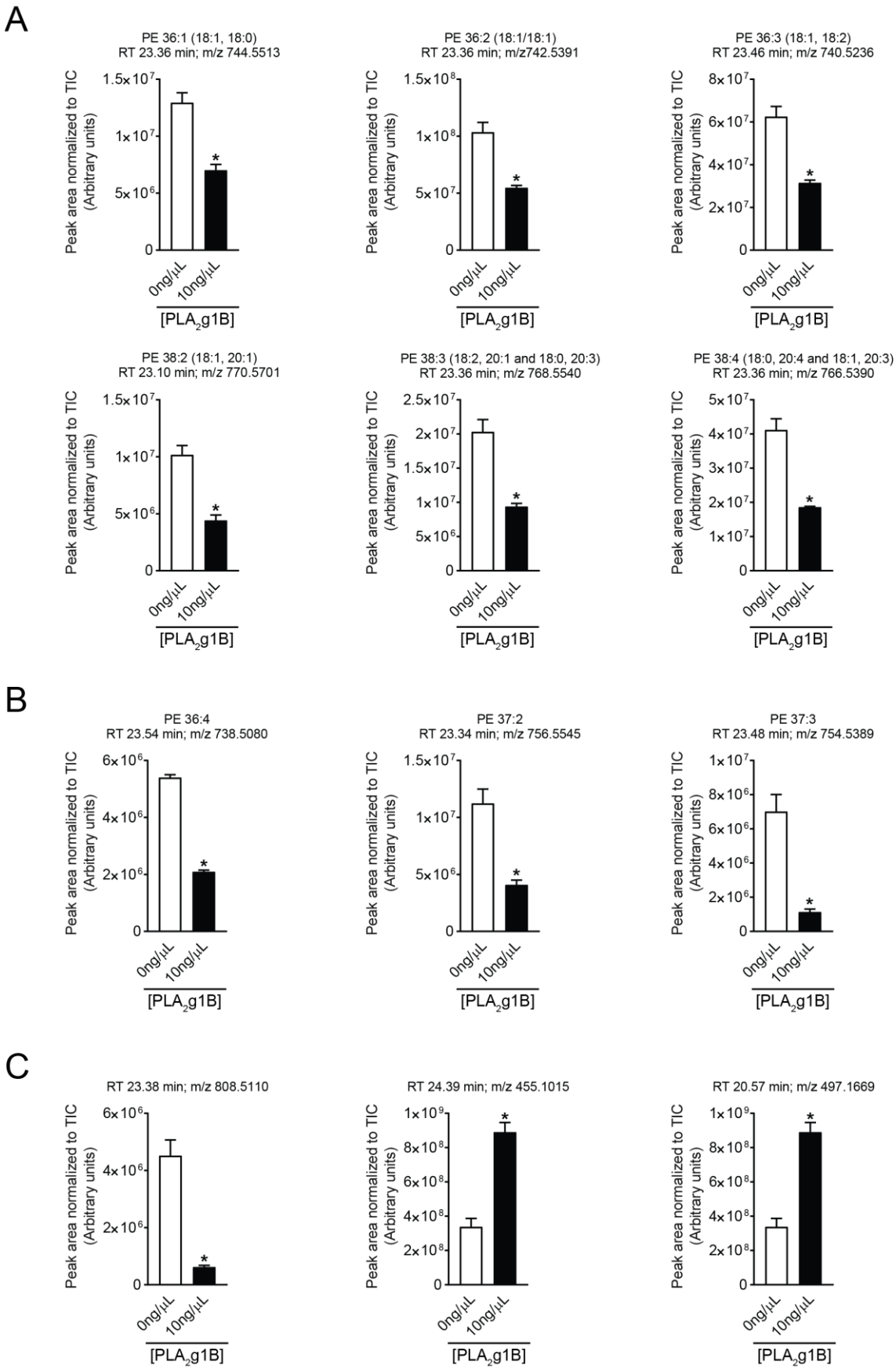

A
PE 36:1 (18:1, 18:0)
RT 23.36 min; m/z 744.5513
PE 36:2 (18:1/18:1)
RT 23.36 min; m/z742.5391
PE 36:3 (18:1, 18:2)
RT 23.46 min; m/z 740.5236
PE 38:2 (18:1, 20:1)
RT 23.10 min; m/z 770.5701
PE 38:3 (18:2, 20:1 and 18:0, 20:3)
RT 23.36 min; m/z 768.5540
PE 38:4 (18:0, 20:4 and 18:1, 20:3)
RT 23.36 min; m/z 766.5390
Peak area normalized to TIC (Arbitrary units)
0ng/μL
10ng/μL
[PLA2g1B]
B
PE 36:4
RT 23.54 min; m/z 738.5080
PE 37:2
RT 23.34 min; m/z 756.5545
PE 37:3
RT 23.48 min; m/z 754.5389
C
RT 23.38 min; m/z 808.5110
RT 24.39 min; m/z 455.1015
RT 20.57 min; m/z 497.1669

Figure 3.17 PLA_2g1B treatment induces changes in L3 *H. polygyrus* larval lipid abundance

Relative abundances of phosphatidylethanolamine (PE) and other unidentified lipids extracted from PLA_2g1B-treated (10 ng/µL) and control-treated (0 ng/µL) larvae. (**A**) Identified PEs. Features were regarded as 'identified' by comparison of their precursor ion and MS/MS fragments with the LipidBlast library, as outlined in **Figure 3.16**. The arrangement of fatty acid moieties on the glycerol backbone (i.e. whether in the *sn*-1 or *sn*-2 position) and the position of double bonds could not be inferred. PE 38:3 and PE 38:4 were detected as a mixture of different fatty acid moieties. (**B**) Putatively identified PEs. The features could be 'annotated' as PEs by comparison of peak retention time and inter-cluster mass shifts of 28 Da (CH_2CH_2) and intra-cluster mass shifts of 2 Da (indicative of difference in double bond number (fatty acid saturation)) with other, identified PEs. MS/MS could not be performed due to low abundance. (**C**) Unidentified lipid compounds. Apolar features that could not be identified, even putatively, by comparison to common libraries. Data is shown as normalized intensities expressed in arbitrary units, n=3 Error bars indicate minimum and maximum values. TIC: Total ion current. RT: Retention time. LC-MS/MS experiments were performed and analysed by Mariana Silva dos Santos and James MacRae.

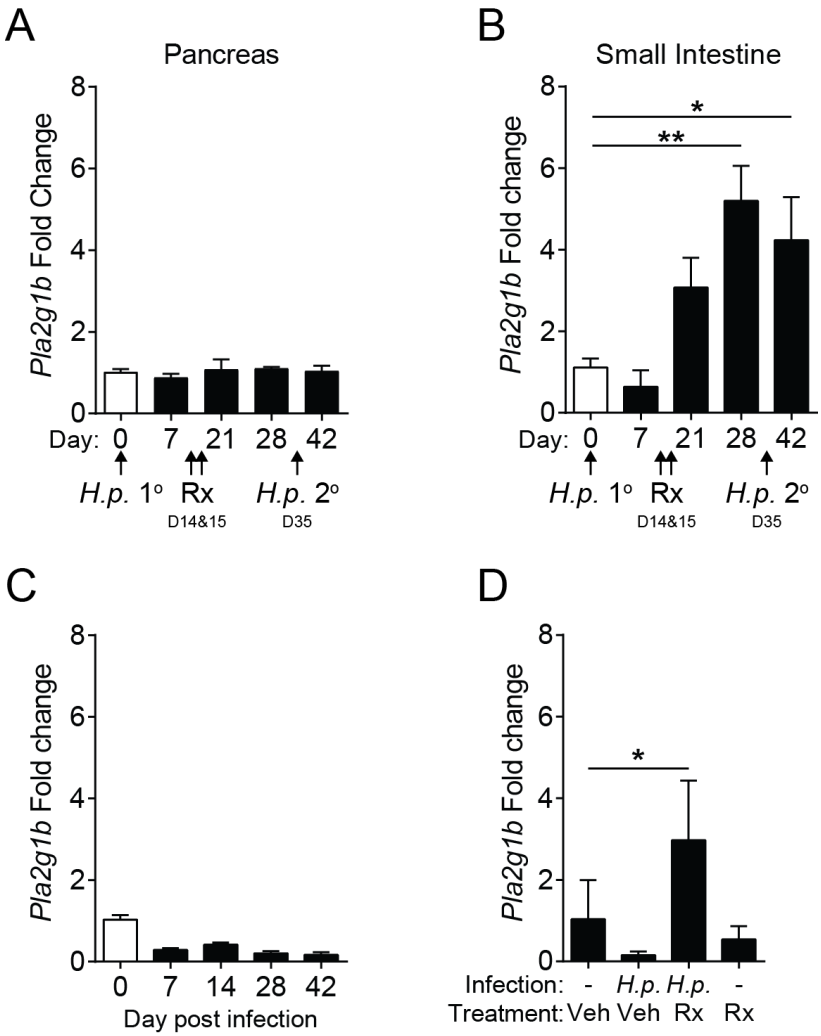


Figure 3.18 *Pla2g1b* expression is induced after drug-clearance of 1° *H. polygyrus* infection

Kinetics of *Pla2g1b* expression in the pancreas (**A**) and small intestine (**B**) during *H. polygyrus* 2° infection model. (**C**) Kinetics of *Pla2g1b* expression in the small intestine during *H. polygyrus* 1° infection. (**D**) *Pla2g1b* expression at day 28 (14 days post Rx) in the small intestine. *H.p.* 1° = 1° *H. polygyrus* infection, *H.p.* 2° = 2° *H. polygyrus* infection, Rx = drug treatment, Veh = vehicle. Data represented as mean ± SEM, n=5-6. All data is representative of at least two independent experiments. * = $p<0.05$, ** = $p<0.01$, determined using a one-way ANOVA with Dunnett's multiple comparison analysis or unpaired two-tailed t test.

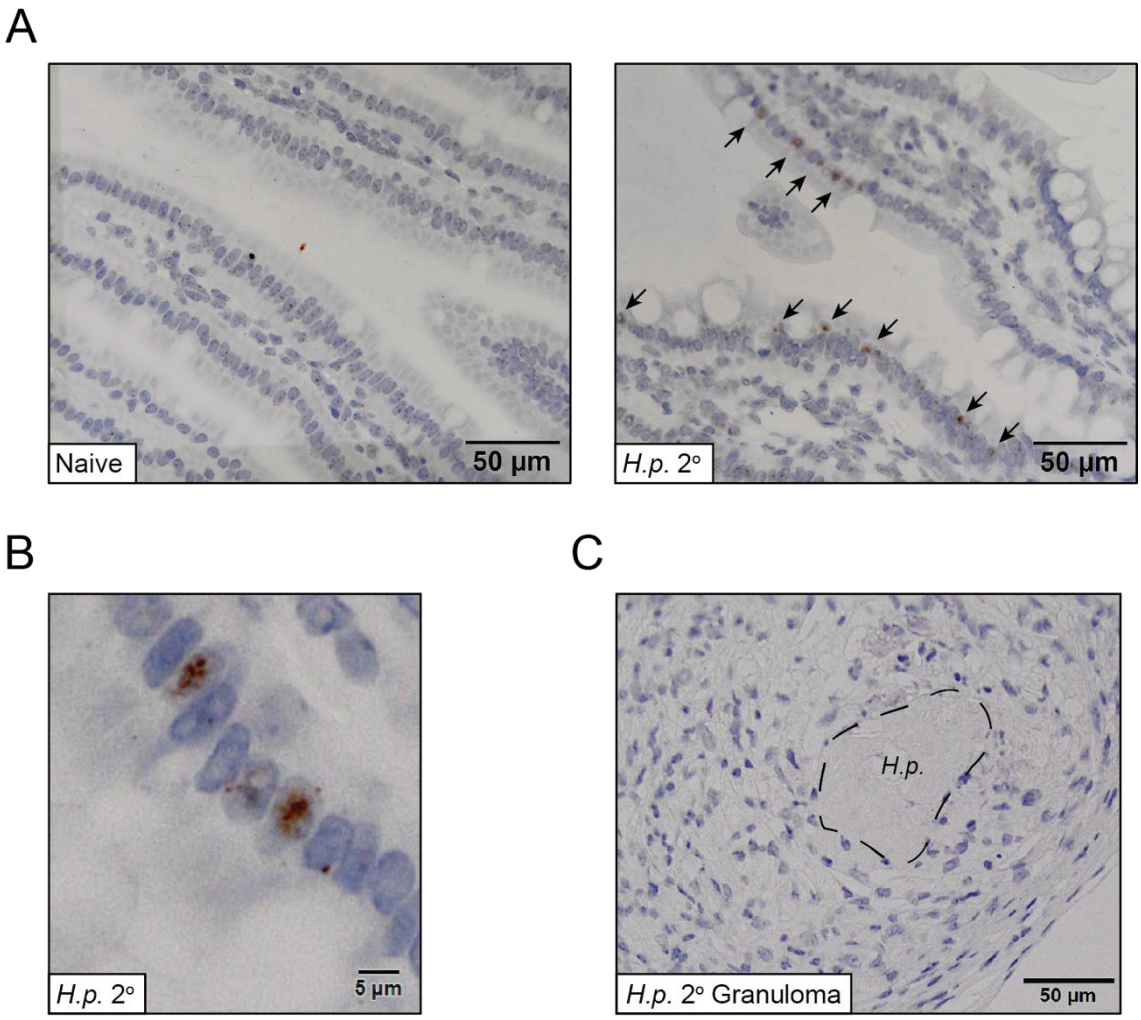

A
Naive
50 µm
H.p. 2°
50 µm
B
H.p. 2°
5 µm
C
H.p.
H.p. 2° Granuloma
50 µm
D
CD45-EpCam+
CD45+EpCam-
Pla2g1b (rel. to Hprt)
0.3
0.2
0.1
0.0
*
ns
Naive
Resistant

Figure 3.19 *Pla2g1b* expression is restricted to epithelial cells in the small intestine of resistant mice

(**A**) *Pla2g1b* detection by RNAScope® ISH in the small intestine of naïve and 7 days-post 2º *H.* polygyrus (*H.p.* 2º) infected mice. (**B**) *Pla2g1b* detection by RNAScope® ISH in epithelial cells of the small intestine 7 days-post 2º *H.* polygyrus infection (*H.p.* 2º). (**C**) *Pla2g1b* detection by RNAScope® ISH in the granuloma surrounding *H. polygyrus* (*H.p.*) within the small intestine 7 days-post 2º *H.* polygyrus infection. (**D**) *Pla2g1b* expression in fluorescence activated cell sorted (FACS) $CD45^{-}EpCam^{+}$ and $CD45^{+}EpCam^{-}$ cells from the intestinal epithelium from naïve and resistant (day 28) mice, n=6. Data represented as mean ± SEM, ns = not significant, * = $p<0.05$ determined using a Mann-Whitney test.

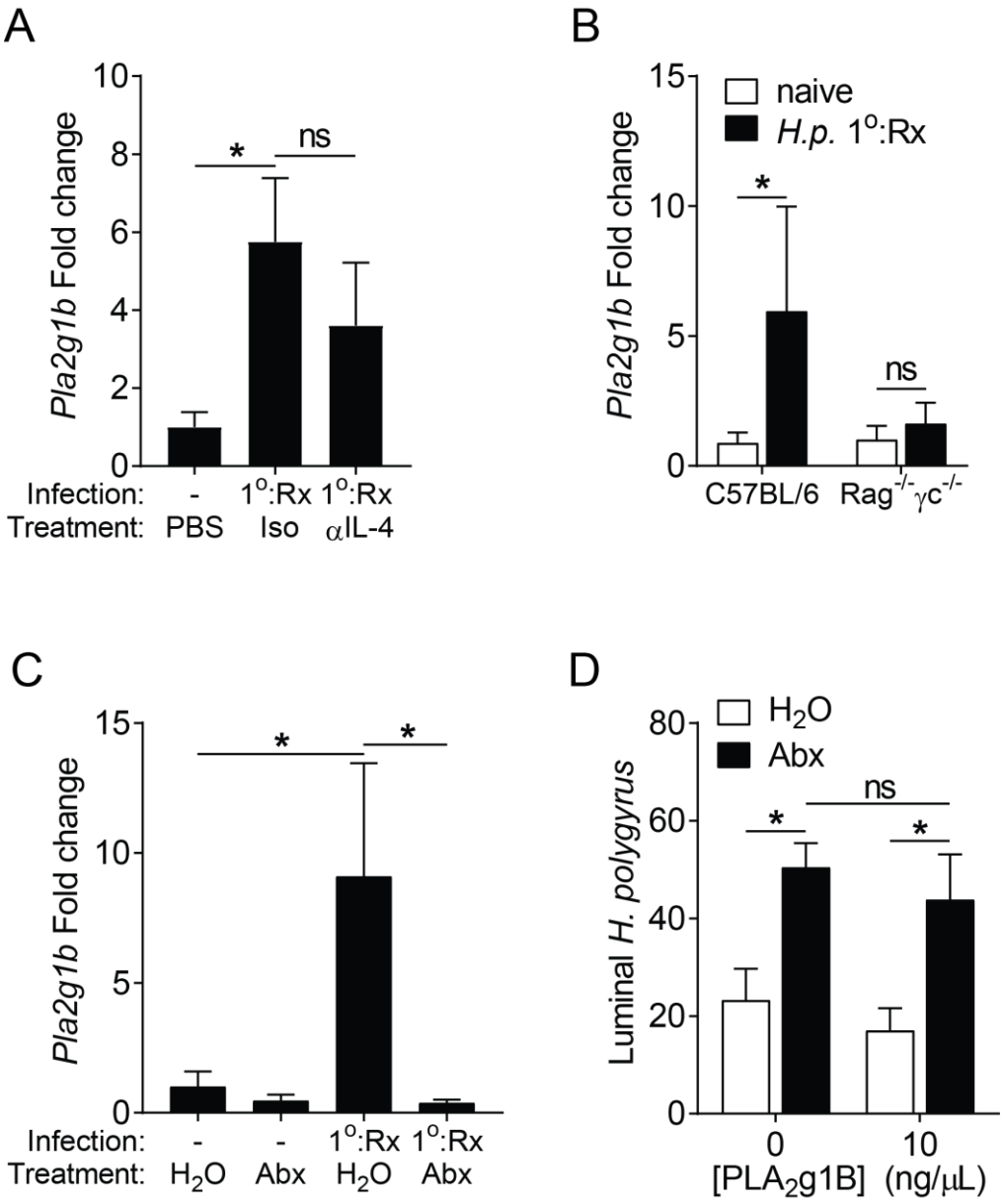
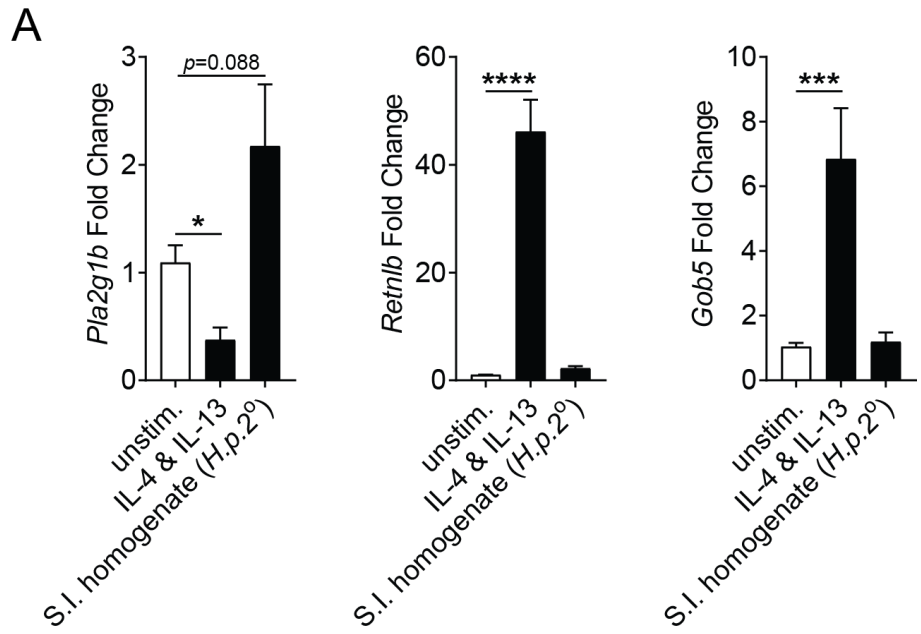


Figure 3.20 Intestinal *Pla2g1b* is regulated by the microbiota and Rag- and common gamma-chain-dependent cells in resistant mice

(**A**) *Pla2g1b* expression at day 28 (14 days-post Rx, 1°-Rx) in the small intestine following anti-IL-4 (αIL-4) or Isotype (Iso) treatment, n=9-10 (data pooled from two independent experiments). (**B**) *Pla2g1b* expression at day 28 (14 days-post Rx, 1°-Rx) in the small intestine, n=7-10 (data pooled from two independent experiments) (**C**) *Pla2g1b* expression at day 28 (14 days post Rx, 1°-Rx) in the small intestine following antibiotic treatment, n=9-10 (data pooled from two independent experiments). (**D**) Luminal *H. polygyrus* worms in the small intestine 14 days-post 2° infection following antibiotic treatment and 24-hour larvae treatment with PLA_2g1B, infective dose = 64 adult worms (determined by 1° infection), n=5, data representative of one experiment. All data represented as mean ± SEM, * = p<0.05, ** = p<0.01, determined using a one-way ANOVA with Tukey's multiple comparison analysis or Mann-Whitney test.


A
Pla2g1b Fold Change
p=0.088
*
Retnlb Fold Change
****
Gob5 Fold Change
***
unstim.
IL-4 & IL-13
S.I. homogenate (H.p.2°)

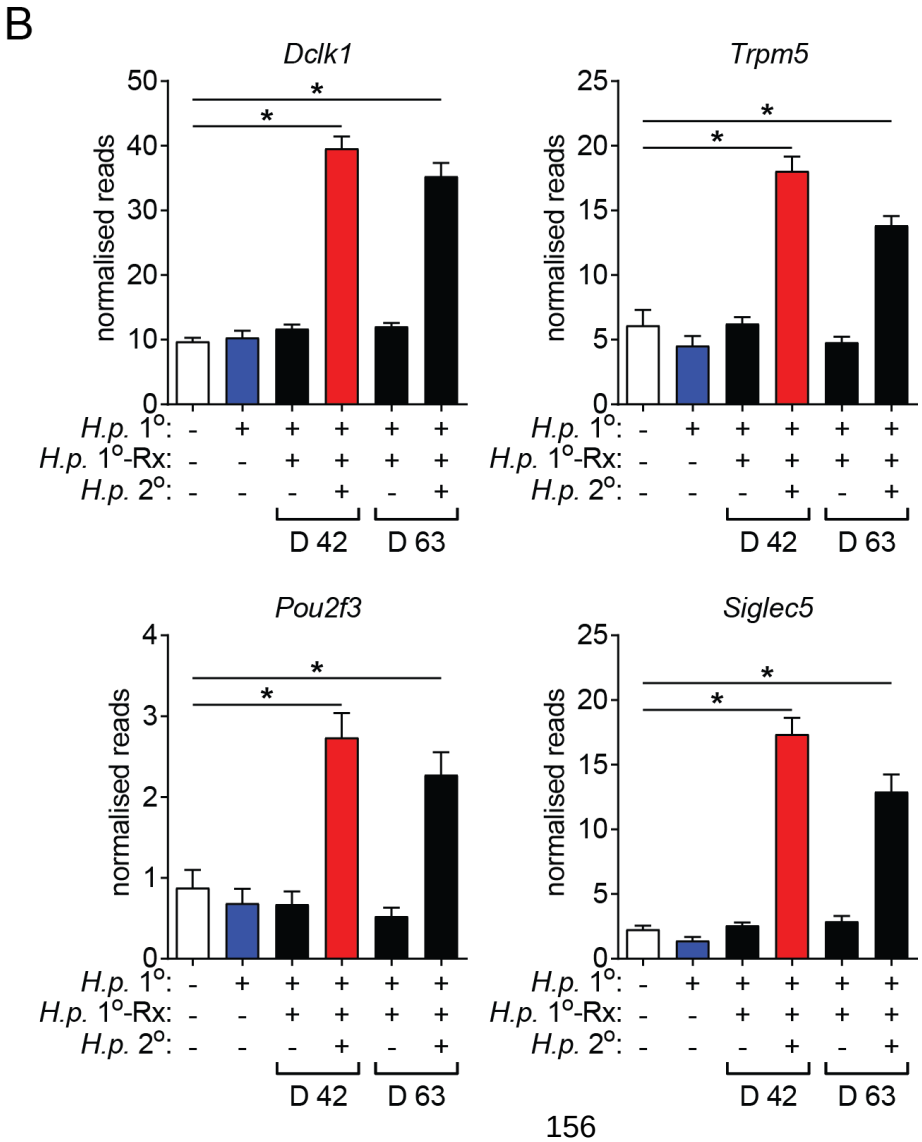

B
Dclk1
Trpm5
Pou2f3
Siglec5
normalised reads
H.p. 1°:
H.p. 1°-Rx:
H.p. 2°:
D 42
D 63

Figure 3.21 *Pla2g1b* expression is negatively regulated by IL-4Rα signalling in intestinal organoids

(**A**) *Pla2g1b, Retnlb* and *Gob5* expression in intestinal organoid cultures following stimulation with IL-4 & IL-13 and small intestinal (S.I.) homogenate from mice 7 days-post 2^{o} *H. polygyrus* infection (*H.p.* 2^{o}). (**B**) Tuft cell-specific gene expression data in small intestine from RNA sequencing data from **Figure 3.1**. Data represented as mean ± SEM, n=. All data is representative of at least three independent experiments. * = $p<0.05$, *** = $p<0.001$, **** = $p< 0.0001$ determined using an unpaired t-test or by RNA sequencing analysis.

Cluster 1: Genes	***H.p.* 2º vs *H.p.* 1º Fold change**	***H.p.* 1º Fold change (rel. to naïve)**	***H.p.* 2º Fold change (rel. to naïve)**
Chi3l3/Chi3l4	31.6	126.6	3995.5
Gsdmc	17.3	67.5	1165.4
Spp1	12.7	5.0	63.8
Rnase2	12.1	373.8	4535.5
Retnlb	7.9	66.0	519.6
Ccl8	7.8	11.6	90.7
Pla2g4c	7.8	49.4	384.1
Retnla	7.5	73.3	547.0
Arg1	5.5	24.0	131.8
Timp1	5.2	11.4	60.0
Olr1	5.1	5.5	28.1
Ccl24	4.3	14.5	62.9
Irg1	3.6	18.0	5.0
Ccl7	3.6	8.4	30.4
F7	3.3	25.1	83.2
Cpxm1	3.2	5.2	16.5
Ccl2	2.6	8.5	21.7
Hp	2.5	12.5	30.8

Ear2 (Includes Others)	2.4	5.1	12.5
F13a1	2.3	5.5	12.5
Trem2	2.2	8.2	18.3
Cbr2	2.2	10.5	23.1
Serpina3	2.1	8.4	17.4
C3	2.0	5.2	10.4
Saa3	2.0	9.8	19.2
2310042E22Rik (Includes Others)	1.7	11.8	20.4
Scd2	1.3	5.7	4.3
Sell	1.1	8.7	9.1

Table 3.1 Cluster 1 gene list

Genes in cluster 1 as determined in **Figure 3.1.D** (Filtered on >5 fold in *H.p.* 1^{o} (relative to naïve), $p<0.05$).

Cluster 2: Genes	***H.p.* 2º vs *H.p.* 1º Fold change**	***H.p.* 1º Fold change (rel. to naïve)**	***H.p.* 2º Fold change (rel. to naïve)**
1810009j06Rik/ Gm2663	162.1	1.0	162.1
Mcpt1	74.3	1.0	74.3
Mcpt2	59.7	1.0	59.7
Reg1b	45.7	1.0	45.7
Reg3d	33.9	1.0	33.9
Cpa3	32.8	1.0	32.8
Spta1	28.7	1.0	28.7
Mir-675	26.3	1.0	26.3
Fetub	22.7	1.0	22.7
Rab44	19.1	1.0	19.1
Ppy	17.5	1.0	17.5
Slc7a2	17.0	1.0	17.0
Ranbp3l	16.9	1.0	16.9
Pah	16.5	1.0	16.5
Cuzd1	16.2	1.0	16.2
Itih4	15.6	1.0	15.6
Prss3	14.7	1.0	14.7
Sprr2g	12.9	1.0	12.9
Amy2a	12.7	1.0	12.7

Prss2	12.6	1.0	12.6
Paqr9	12.4	1.0	12.4
Try4/Try5	12.4	1.0	12.4
Dcdc2	12.2	1.0	12.2
Gc	11.9	1.0	11.9
Tmed11	11.4	1.0	11.4
Ggh	11.2	1.0	11.2
Sycn	11.1	1.0	11.1
Cel	11.1	1.0	11.1
Serpini2	10.9	1.0	10.9
Amy1a (Includes Others)	10.8	1.0	10.8
Ccl2	10.6	1.0	10.6
Klk3	10.6	1.0	10.6
Gp2	10.1	1.0	10.1
Cela3a	9.9	1.0	9.9
Ptf1a	9.8	1.0	9.8
Sostdc1	9.8	1.0	9.8
Ctrb2	9.7	1.0	9.7
Cpa1	9.7	1.0	9.7
Tmed6	9.6	1.0	9.6

Cckar	9.6	1.0	9.6
Gm5409/Try10	9.6	1.0	9.6
Cldn10	9.1	1.0	9.1
Aass	8.7	1.0	8.7
Rnase1	8.6	1.0	8.6
Ptger3	8.2	1.0	8.2
Slc38a3	8.2	1.0	8.2
Pnliprp2	8.0	1.0	8.0
Cbs	7.9	1.0	7.9
Retnlb	7.9	1.0	7.9
Amy2b	7.8	1.0	7.8
Alox15	7.8	1.0	7.8
Clps	7.8	1.0	7.8
Loc100862462	7.7	1.0	7.7
Siglec5	7.7	1.0	7.7
Gatm	7.6	1.0	7.6
Erp27	7.6	1.0	7.6
Angpt1	7.5	1.0	7.5
Slc34a2	7.5	1.0	7.5
Tnip3	7.5	1.0	7.5
Muc6	7.5	1.0	7.5

Cpb1	7.4	1.0	7.4
C8orf47	7.4	1.0	7.4
Casp9	7.3	1.0	7.3
Sh2d6	7.3	1.0	7.3
Pla2g1b	7.3	1.0	7.3
Lrrc7	7.2	1.0	7.2
Hapln4	7.1	1.0	7.1
Igk	7.1	1.0	7.1
Chst2	7.1	1.0	7.1
Nupr1	6.8	1.0	6.8
Rgs22	6.8	1.0	6.8
Vtn	6.7	1.0	6.7
Pnliprp1	6.7	1.0	6.7
Aqp12a/Aqp12b	6.6	1.0	6.6
Gabra4	6.6	1.0	6.6
Iapp	6.6	1.0	6.6
Lrrn1	6.5	1.0	6.5
Ighg2c	6.4	1.0	6.4
Arhgdig	6.3	1.0	6.3
Hamp/Hamp2	6.3	1.0	6.3
Igf1	6.3	1.0	6.3

Tmem108	6.3	1.0	6.3
Tff2	6.3	1.0	6.3
Wfdc18	6.3	1.0	6.3
Ang2 (Includes Others)	6.2	1.0	6.2
Treml2	6.2	1.0	6.2
Ly6g6f	6.1	1.0	6.1
Clu	6.1	1.0	6.1
Ctsv	6.1	1.0	6.1
Sh2d7	6.1	1.0	6.1
Cela3b	6.1	1.0	6.1
Cilp	5.9	1.0	5.9
Alox5ap	5.9	1.0	5.9
Nphs1	5.9	1.0	5.9
Gnmt	5.9	1.0	5.9
Dhrs9	5.9	1.0	5.9
Lcat	5.9	1.0	5.9
Fut2	5.9	1.0	5.9
Prss1	5.9	1.0	5.9
Aldh1l2	5.8	1.0	5.8
Gprc5c	5.7	1.0	5.7

Fkbp11	5.7	1.0	5.7
Muc1	5.7	1.0	5.7
Rny1	5.7	1.0	5.7
Hck	5.7	1.0	5.7
Tfpi2	5.6	1.0	5.6
G6pc	5.6	1.0	5.6
Igh-VS107	5.6	1.0	5.6
Pdcd1lg2	5.6	1.0	5.6
Cpa2	5.5	1.0	5.5
Hpn	5.5	1.0	5.5
Clec7a	5.5	1.0	5.5
Gal	5.4	1.0	5.4
Rbpjl	5.3	1.0	5.3
Nfil3	5.3	1.0	5.3
Srgn	5.2	1.0	5.2
Cldn8	5.2	1.0	5.2
Fam221a	5.2	1.0	5.2
Hsbp1l1	5.2	1.0	5.2
Cela1	5.1	1.0	5.1
Me1	5.1	1.0	5.1
Ca4	5.1	1.0	5.1

Tspan6	5.1	1.0	5.1
Fn1	5.1	1.0	5.1
Slc38a5	5.1	1.0	5.1
Glipr2	5.0	1.0	5.0

Table 3.2 Cluster 2 gene list

Genes in cluster 2 as determined in **Figure 3.1.E** (Filtered on >5 fold in *H.p.* 2^{o} (relative to naïve), $p<0.05$).

Cluster 3: Genes	***H.p.* 2º vs *H.p.* 1º Fold change**	***H.p.* 1º Fold change (rel. to naïve)**	***H.p.* 2º Fold change (rel. to naïve)**
Gm11194	-33.2	1.0	-33.2
Slc13a2	-20.6	1.0	-20.6
Dbp	-11.1	1.0	-11.1
Cyp2a12/ Cyp2a22	-7.6	1.0	-7.6

Table 3.3 Cluster 3 gene list

Genes in cluster 3 as determined in **Figure 3.1.E** (Filtered on <-5 fold in *H.p.* 2º (relative to naïve), $p<0.05$).

Chapter 4. Results 2: The role of microRNAs in antihelminth immunity

4.1 Introduction

microRNAs (miRNAs) are small, non-protein coding RNA molecules of approximately 21 nt in length, which negatively regulate mRNA translation. miRNAs can act by either translational inhibition or mRNA degradation (Ambros, 2003), thus providing finite control over gene expression. As a result, miRNAs have been implicated in a plethora of biological settings, from development to immunity.

In the nucleus, miRNAs are transcribed from their respective gene by RNA polymerase II to produce primary miRNA (pri-miRNA) molecules (Lee et al., 2004, Lee et al., 2002). The pri-miRNA molecule is then recognised by the RNA binding protein Dgcr8, recruiting the RNase Drosha to allow the pri-miRNA molecule to be processed to form a precursor miRNA (pre-miRNA) molecule (Lee et al., 2003, Han et al., 2004, Denli et al., 2004, Gregory et al., 2004, Landthaler et al., 2004). Exportin-5 facilitates the transport of the pre-miRNA out of the nucleus and into the cytoplasm (Yi et al., 2003). In the cytoplasm the pre-miRNA is processed into mature, functional miRNA molecules by the RNase Dicer (Hutvagner et al., 2001, Ketting et al., 2001, Knight and Bass, 2001). The free mature miRNA molecule is then recognised and bound by an Argonaute (Ago) protein to form a miRNA-Ago complex (Chendrimada et al., 2005). This complex is then incorporated into the RNA-induced silencing complex (RISC), where the miRNA guides the RISC to its complementary mRNA target(s) for gene silencing, preventing mRNA translation into protein (Maniataki and Mourelatos, 2005, MacRae et al., 2005, Gregory et al., 2005, Martinez et al., 2002).

Animal models of intestinal helminth infections support the notion that type 2 poalrised immune responses are essential for protective immunity (Reynolds et al., 2012, Maizels et al., 2012b, Allen and Maizels, 2011). miRNAs have been investigated in a variety of immunological processes and are essential in the resolution of many infectious diseases. Despite this, the identification and role of miRNAs in immunity to intestinal helminth infections is relatively unstudied.

In this Chapter, we set out to identify and test the role of miRNAs in intestinal helminth infection. Specifically asking how local miRNA expression changes following helminth infection and identifying miRNAs that are essential for antihelminth immunity. To answer these questions, we utilised the naturally occurring, strictly enteric, murine intestinal helminth *H. polygyrus* as it enabled us to model susceptibility and resistance in C57BL/6 mice using the 2^{o} challenge infection model (Finkelman et al., 1997), as described above. We employed genetic tools to induce global miRNA downregulation *in* vivo, broadly investigating the role of miRNAs in immunity to intestinal helminth infection. We also took a more precise approach, utilising small sequencing of small intestinal tissue from mice both susceptible and resistant to *H. polygyrus*, as described in **Chapter 3**, to identify miRNAs implicated in immunity to intestinal helminths. Coupling both our miRNA and mRNA sequencing datasets, we were able to elucidate candidate miRNAs and putative mRNA targets, essential for resistance to *H. polygyrus.*

4.2 Results

4.2.1 Global downregulation of miRNA species does not induce resistance to *H. polygyrus*

Previous studies have demonstrated the importance of a memory type 2 immune response in mediating resistance to 2^{o} *H. polygyrus* challenge infection (Katona et al., 1991, Urban et al., 1991a, Urban et al., 1991b). A mixed type 2 and potent regulatory immune response is mounted upon 1^{o} infection, allowing a chronic infection to establish (Reynolds et al., 2012). However, the type 2 immune response initiated upon 2^{o} infection occurs more quickly and at a greater magnitude (Pelly et al., 2017, Morimoto et al., 2004), also supported by our mRNA sequencing data (**Figure 3.1** and **Figure 3.2**).

In light of this, we hypothesised that miRNAs provide another level of regulation, inhibiting mRNA translation and therefore protein production, restraining protective type 2 immune responses during a 1^{o} *H. polygyrus* infection. We also hypothesised that downregulation of miRNAs would release this regulatory ‘brake’ on the immune

system, permitting a robust effector response to develop during a 2^{o} *H. polygyrus* infection.

To test this hypothesis, we generated an inducible system in mice where we could eliminate mature miRNA production *in vivo* during a 1^{o} *H. polygyrus* infection, in an attempt to induce resistance. Constitutive *Dicer*-deficient mice are embryonically lethal (Bernstein et al., 2003). We therefore crossed the *Dicer*$^{fl/fl}$ mice with the mice containing the ubiquitous inducible Cre-recombinase driver *R26*^{CreERT2}. Following tamoxifen treatment, the *R26*^{CreERT2}*Dicer*$^{fl/fl}$ mouse will delete *Dicer,* the enzyme required for mature miRNA production (Hutvagner et al., 2001, Ketting et al., 2001, Knight and Bass, 2001).

4.2.1.1 Inducible *Dicer* deletion reduced mature miRNAs in the small intestine

To confirm that the mouse functioned as expected, we confirmed that tamoxifen treatment *in vivo* would remove *Dicer* expression and reduce mature miRNA expression. We crossed the *R26*^{CreERT2}*Dicer*$^{fl/fl}$ mice with the *R26*^{yfp}*Dicer*$^{fl/fl}$ mice to produce the *R26*$^{CreERT2/yfp}$*Dicer*$^{fl/fl}$ mice. This provided two functions: firstly, it allowed us to track Cre-recombinase activity. Secondly, it kept the *R26*^{CreERT2} allele heterozygous as homozygosity for this allele can induce Cre-recombinase toxicity upon tamoxifen administration (*personal communication with E. Scheighoffer, The Francis Crick Institute*). *R26*$^{CreERT2/yfp}$*Dicer*$^{fl/fl}$ mice were treated with either tamoxifen or vehicle only for 5 consecutive days (days 0, 1, 2, 3 and 4) and we harvested the small intestinal RNA at day 7 (**Figure 4.1.A**). Tamoxifen treatment led to a 50% reduction in *Dicer* mRNA expression (**Figure 4.1.B**) and a 50-60% reduction in mature miRNA expression (**Figure 4.1.C**). Despite imperfect deletion, we then investigated the effect of this miRNA reduction on immunity to *H. polygyrus* infection.

4.2.1.2 Reduced *Dicer* expression does not impact resistance to *H. polygyrus*

To test whether the 50-60% reduction of mature miRNA expression had any impact on immunity to *H. polygyrus*, we administered tamoxifen for 5 consecutive days (days -5, -4, -3, -2 and -1) prior to infection with *H. polygyrus*. We harvested the small

intestinal tissue 14 days-post infection to confirm *Dicer* deletion and determine worm burden (**Figure 4.2.A**). We again identified a 50% decrease in *Dicer* expression 14 days-post infection (15 days-post final tamoxifen treatment) (**Figure 4.2B**), similar to that previously seen 3 days-post final tamoxifen treatment (**Figure 4.1B**), indicating that *Dicer* competent cells had not repopulated over the course of infection. Despite the 50% decrease in *Dicer* expression there was no significant change in luminal *H. polygyrus* adult worms or eggs secreted in the faeces (**Figure 4.2.C-D**), suggesting that 50% downregulation in mature miRNAs did not permit resistance to 1^{o} *H. polygyrus* infection.

For technical reasons, we could not test our hypothesis with these genetic tools. From the data generated, with 50-60% reduction of Dicer and miRNAs, we rejected our hypothesis outlined in **4.2.1**, that miRNAs restrain protective responses to 1^{o} *H. polygyrus* infection and that downregulation of all mature miRNAs restrict resistance to 1^{o} *H. polygyrus* infection. These studies suggest that the regulation of host immunity to *H. polygyrus* infection is likely more complex than global down- or global upregulation of miRNA species. As a result, to further understand the roles of specific miRNA species mediating immunity to intestinal helminth infection we performed miRNA sequencing of the small intestinal tissue of mice susceptible and resistant to *H. polygyrus*, in the same model utilised in **Chapter 3**, to identify miRNAs implicated in immunity to *H. polygyrus*.

4.2.2 Intestinal miRNA expression in mice resistant to *H. polygyrus*

4.2.2.1 miRNA sequencing identifies 24 candidate miRNAs implicated in resistance to *H. polygyrus* infection

We infected a large cohort of C57BL/6 mice with *H. polygyrus* L3 larvae. A group of mice were sacrificed at day 7 post 1^{o} infection (*H.p.* 1^{o}). The remaining mice were drug cured (Rx) of the 1^{o} infection on days 14 and 15. Some mice were culled on days 42 and 63 after drug-cure (Rx (D42) and Rx (D63), respectively). Other mice were 2^{o} challenge infected on day 35 or day 56 and sacrificed 7 days later (*H.p.* 2^{o} (D42) and *H.p.* 2^{o} (D63), respectively). At each time point, duodenal tissue was dissected and the RNA extracted for mRNA and miRNA sequencing (**Figure 4.3.A**).

This was the same experiment previously described in **Chapter 3**, however we took a portion of the RNA for miRNA sequencing. This enabled us to have mRNA and miRNA transcriptomes for each animal in each group.

Of note, the depth of the miRNA sequencing performed in these experiments was sub-optimal. Ideally, a sequencing depth of 12 million reads per sample is optimal, allowing detection approximately 80% of all expressed miRNAs (Sun et al., 2014). We only achieved 1-3 million reads per sample and therefore lowly expressed miRNA species may not have been detected. In addition, only 4 biological replicates were used for the miRNA sequencing, unlike the 8 replicates used for mRNA sequencing. This was due to technical difficulties in creating the miRNA sequencing libraries. As a result, any candidate miRNAs identified with miRNA sequencing had to be validated by qRT-PCR using the full 8 biological replicates.

Following miRNA sequencing of the duodenal tissue we only identified 5 miRNA species significantly differentially expressed in *H.p.* 1^{o} (relative to naïve, p<0.05). This number was increased after drug-cure to 16 and 19 differentially expressed miRNAs (relative to naïve, p<0.05), Rx (D42) and Rx (D63) respectively. However, the highest number of differentially expressed miRNAs was seen after 2^{o} infection, with 35 and 67 miRNAs significantly differentially expressed (relative to naïve, p<0.05), *H.p.* 2^{o} (D42) and *H.p.* 2^{o} (D63) respectively (**Figure 4.3.B**).

To identify miRNAs that may influence resistance to *H. polygyrus*, we compared the miRNA transcriptome of *H.p.* 1^{o}, *H.p.* 2^{o} (D42) and *H.p.* 2^{o} (D63) (each relative to naïve, p<0.05) (**Figure 4.3.C**). This comparison identified 22 miRNAs that were qualitatively different between susceptible and resistant mice, not expressed in *H.p.* 1^{o} but were expressed in both early *H.p.* 2^{o} (D42) and later *H.p.* 2^{o} (D63). We also identified 2 miRNAs that were commonly expressed in *H.p.* 1^{o}, *H.p.* 2^{o} (D42) and *H.p.* 2^{o} (D63) (relative to naïve, p<0.05), but were quantitatively different in resistant mice, with increased expression in both *H.p.* 2^{o} (D42) and *H.p.* 2^{o} (D63) (**Figure 4.3.D**). Utilising both early and late time points of *H.p.*2^{o} infection increased our confidence that these miRNAs may influence resistance to infection.

Overall, miRNA sequencing identified 24 candidate miRNAs differentially expressed in resistant mice, compared to susceptible and naïve mice.

4.2.2.2 qRT-PCR validation identifies miR-99a-5p, miR-148a-3p and miR-155-5p to be upregulated in mice resistant to *H. polygyrus*.

miRNA sequencing identified 24 candidate miRNAs implicated in resistance to *H. polygyrus* infection. These 24 candidate miRNAs were then validated by qRT-PCR in the full 8 biological replicates. The criteria for candidate miRNA selection was as follows; miRNA expression must be statistically significant ($p<0.05$) in both *H.p.* 2^{o} (D42) and *H.p.* 2^{o} (D63), when compared to naïve. Using these criteria three candidate miRNAs, miR-99a-5p, miR-148a-3p and miR-155-5p, were validated and taken forward for further analysis (**Figure 4.4.A**). For all three candidate miRNAs, qRT-PCR validation closely resembled miRNA sequencing expression, confirming two different expression dynamics. Both miR-99a-5p and miR-148a-3p expression was unaltered between naïve and *H.p.* 1^{o} but were significantly upregulated following drug treatment and remained elevated with or without 2^{o} challenge infection (**Figure 4.4.A**). In contrast, miR-155-5p expression was only induced upon 2^{o} challenge infection (**Figure 4.4.A**).

Of the remaining miRNAs. 6 were significantly differentially expressed in either *H.p.* 2^{o} (D42) or *H.p.* 2^{o} (D63) ($p<0.05$), compared to naïve mice, and therefore not taken forward for further analysis (**Figure 4.4.B**). One other miRNA, miR-326-3p, was significantly differentially expressed in both *H.p.* 2^{o} (D42) and *H.p.* 2^{o} (D63) ($p<0.05$, compared to naïve), but the qPCR and RNA sequencing expression profile differed drastically. For this reason, miR-326-3p was not taken forward for further analysis (**Figure 4.4.C**). The remaining miRNAs were not significantly differentially expressed in either *H.p.* 2^{o} (D42) or *H.p.* 2^{o} (D63) ($p<0.05$, compared to naïve).

Of the three candidate miRNAs, only miR-155 has been shown to be essential for immunity to *H. polygyrus*, with resistance to 2^{o} *H. polygyrus* abrogated in miR-155$^{-/-}$ mice (Okoye et al., 2014). miR-99a-5p and miR-148a-3p have not been implicated in antihelminth immunity or, more broadly, type 2 immunity. We therefore took these three candidate miRNAs forward, analysing their putative mRNA targets and testing their roles in immunity to *H. polygyrus*.

4.2.3 Complementary mRNA sequencing and analysis identifies candidate miRNA putative mRNA targets

Using the mRNA sequencing dataset (**Figure 3.1**), complementary to the miRNA dataset (**Figure 4.1**), we performed *in silico* 'expression paired analysis' using IPA® software to identify putative mRNA targets that were inversely regulated to the three candidate miRNAs.

From both *H.p.* 2º (D42) and *H.p.* 2º (D63), we identified mRNAs that were significantly differentially expressed (relative to naïve, $p<0.05$) and predicted to be direct targets of either miR-99a-5p, miR-148a-3p or miR-155-5p *in silico* and followed the paired expression profile (*i.e.* mRNA expression increased if miRNA is decreased or, in our case, mRNA expression decreased if miRNA is increased) (**Figure 4.5.A**). Assuming that the mechanisms of immunity are similar between early *H.p.* 2º (D42) and late *H.p.* 2º (D63), we filtered the list of putative mRNA gene targets to those that were common to both *H.p.* 2º (D42) and *H.p.* 2º (D63), and passed a 1.5-fold change filter (**Figure 4.5.B**). We identified distinct regulatory nodes for each candidate miRNA, with very few common mRNA targets shared between the three candidate miRNAs. Unfortunately, the number of mRNA targets for each miRNA was too small to adequately perform pathway analysis functions, therefore preventing the identification of miRNA-regulated pathways that contribute to immunity against *H. polygyrus*.

4.2.4 Candidate miRNA inhibition *in vivo*

4.2.4.1 Pharmacological inhibition of miR-99a-5p, miR-148a-3p and miR-155-5p does not perturb resistance to *H. polygyrus* infection

To test whether elevated miR-99a-5p, miR-148a-3p or miR-155-5p were required for resistance to *H. polygyrus*, we used pharmacological miRNA inhibitors during 2º challenge infection (**Figure 4.6.A**). The locked nucleic acid (LNA) miRNA inhibitors act by competitively binding to the target miRNA. Inhibitors were used for two main reasons. Firstly, to our knowledge, miR-99a-5p and miR-148a-3p knockout mice

have not been generated. Secondly, therapeutic and transient inhibition of miRNAs would avoid the impact of miRNA inhibition on any developmental processes.

As all three miRNAs were significantly upregulated 7 days-post 2^{o} challenge infection (**Figure 4.4**), we treated mice with miRNA inhibitors before (day 33), during (day 35) and after (day 37 and 40) 2^{o} *H. polygyrus* infection (**Figure 4.6.A**). At day 49 (14 days after 2^{o} *H. polygyrus* infection) we tested the effect of the miRNA inhibitors on miRNA expression, parasitology and immunity. We identified that all of the individual miRNA inhibitors, miR-99a-5p$^{\Delta}$, miR-148a-3p$^{\Delta}$ and miR-155-5p$^{\Delta}$, significantly reduced the expression of their respective miRNA targets in the small intestine (relative to vehicle treatment) (**Figure 4.6.B**). We also confirmed that the control inhibitor had no significant effect on any candidate miRNA expression (**Figure 4.6.B**).

Control inhibitor-treated mice maintained resistance to *H. polygyrus* infection, with a significant reduction in luminal worm numbers and eggs secreted in the host faeces 14 days-post 2^{o} infection compared to 1^{o} infection (**Figure 4.6.C** and **Figure 4.7.D**). However, individual miRNA inhibition also had no significant effect on resistance to *H. polygyrus* with luminal worm numbers and secreted eggs not significantly different to mice receiving the control inhibitor, despite a small trend in increased luminal worms following miR-148a-3p or miR-155-5p inhibition (**Figure 4.6.C** and **Figure 4.7.D**).

4.2.4.2 Pharmacological inhibition of miR-99a-5p, miR-148a-3p and miR-155-5p does not perturb type 2 immune responses following 2^{o} *H. polygyrus* infection

Pharmacological inhibition of miR-99a-5p, miR-148a-3p or miR-155-5p did not abrogate resistance to *H. polygyrus*. However, we determined whether miRNA inhibition had any impact on immune responses following 2^{o} challenge infection. Th2 cell differentiation and type 2 cytokine production is essential in mediating resistance to *H. polygyrus* (Urban et al., 1995, Urban et al., 1991a, Urban et al., 1991b). The frequency of IL-4-, IL-13- and IL-5-producing CD44^{hi}CD4^{+} cells had significantly increased in the mLN 14 days-post 2^{o} infection in the control inhibitor-treated mice, compared to naïve (**Figure 4.7.A**). Following miRNA inhibitor treatment, a similar

increased frequency of type 2 cytokine-producing $CD4^{+}CD44^{hi}$ cells was observed. No aberrant production of IFNγ or IL-17A was identified (**Figure 4.7.A**). In the spleen, the frequency of IL-4-, IL-13-, IL-5- and IFNγ-producing $CD44^{hi}CD4^{+}$ cells was significantly increased 14 days-post 2^{o} infection in the control inhibitor-treated mice, compared to naïve, and unchanged following miR-99a-5p$^{\Delta}$ or miR-155-5p$^{\Delta}$ (**Figure 4.7.B**). However, we observed a small, but significant decrease in the frequency of IL-4 and IL-5 producing $CD44^{hi}CD4^{+}$ cells following miR-148a-3p$^{\Delta}$, when compared to control inhibitor-treated mice (**Figure 4.7.B**). Despite the decrease in IL-4 and IL-5 producing T cells following miR-148a-3p$^{\Delta}$, this had no impact on resistance to *H. polygyrus* 2^{o} infection (**Figure 4.6.C**).

The type 2 effector immune response, mediated by the Th2 cytokines IL-4 and IL-13, was also intact, with production of *H. polygyrus*-specific IgG1 unaltered following inhibition of either miR-99a-5p, miR-148a-3p or miR-155-5p (**Figure 4.8.A**). Similarly, the induction of *Arg1*, Relmβ *(Retnlb*) and *Gob5* was also unaltered following inhibition of either miR-99a-5p, miR-148a-3p or miR-155-5p (**Figure 4.8.B-C**).

Taken together, these data demonstrate that the inhibition of miR-99a-5p, miR-148a-3p or miR-155-5p did not alter the antihelminth type 2 immunity.

4.2.5 Concurrent candidate miRNA inhibition *in vivo*

4.2.5.1 Simultaneous inhibition of miR-99a-5p, miR-148a-3p and miR-155-5p abrogates resistance to *H. polygyrus*

Individual pharmacological inhibition of miR-99a-5p, miR-148a-3p or miR-155-5p had very little effect on immune responses and no impact on immunity to *H. polygyrus* (**Figure 4.6**, **4.7** and **4.8**). Given that all three miRNAs were simultaneously elevated during resistance (**Figure 4.4.A**), we hypothesised that all three miRNAs may act in concert and that concurrent inhibition of all three miRNAs would abrogate expulsion following a 2^{o} challenge infection.

We therefore treated mice with PBS, control inhibitor or all three miRNA inhibitors combined before (day 33), during (day 35) and after (day 37 and 40) 2^{o} *H. polygyrus*

infection (**Figure 4.6.A**). Control inhibitor treatment had no effect on the expression of miR-99a-5p, miR-148a-3p or miR-155-5p expression in the small intestine, whereas the triple miRNA inhibitor treatment (miRNA$^{3\Delta}$) significantly reduced the expression of all three miRNAs (**Figure 4.9.A**).

As demonstrated previously, control inhibitor treated mice were resistant to 2^{o} *H. polygyrus* infection, with no significant difference seen when compared to vehicle control treated mice (**Figure 4.9.B-C**). However, miRNA$^{3\Delta}$ abrogated resistance to 2^{o} *H. polygyrus* infection, with mice harbouring significantly more luminal worms than control inhibitor treated mice, comparable to that of a 1^{o} infection (**Figure 4.9.B**).

4.2.5.2 Simultaneous inhibition of miR-99a-5p, miR-148a-3p and miR-155-5p induces an inappropriate T cell response

ICS analysis of activated CD4^{+} T cells in the mLN revealed no perturbation in the frequency of type 2 cytokine-producing T cells (IL-4, IL-13 or IL-5) and no aberrant induction of IFNγ- or IL-17A-producing T cells following miRNA$^{3\Delta}$, when compared to control inhibitor treatment (**Figure 4.10.A**). However, ICS analysis in the spleen identified a significant reduction of type 2 cytokine-producing CD44^{hi}CD4^{+} T cells and an increase in IFNγ- and IL-17A-producing CD44^{hi}CD4^{+} T cells following miRNA$^{3\Delta}$ (**Figure 4.10.B**). Th2 cells and type 2 cytokine production is essential for resistance to *H. polygyrus* (Urban et al., 1991a, Urban et al., 1991b) and aberrant Th1 cell differentiation and cytokine production has previously been demonstrated to abrogate protective immunity and promote chronicity in *H. polygyrus* and other intestinal helminth infections (Coomes et al., 2015, Bancroft et al., 1994, Perrigoue et al., 2009). To quantitate the *H. polygyrus*-specific cytokine production by T cells we stimulated the mLN cells, isolated following 2^{o} challenge infection, with *H. polygyrus* antigen *ex vivo*. We identified an increased production of the Th1 cytokine IFNγ following miRNA$^{3\Delta}$ treatment, although not reaching statistical significance, when compared to control inhibitor treatment (**Figure 4.11.A**). The production of the Th2 cytokine IL-13 was intact following *H. polygyrus* antigen stimulation *ex vivo* (**Figure 4.11.A**). The antigen specific *ex vivo* stimulation data (**Figure 4.11.A**) is in discrepancy with the results generated from ICS in the mLN (**Figure 4.10.A**), but in

agreement with the ICS data from the spleen (**Figure 4.10.B**). In the mLN, this may be due the differences between the stimulation, the antigen independent PMA and ionomycin stimulation of all cells or the antigen-specific stimulation of T cells. Furthermore, the ICS data informs of the frequency of cytokine-producing T cells and does not absolutely quantitate the cytokine produced, unlike the absolute cytokine detection by ELISA following antigen-specific stimulation *ex vivo*.

Overall, the T cell cytokine production data is inconclusive, but suggested that a comparable Th2 response was induced following 2^{o} *H. polygyrus* infection of triple miRNA inhibitor treated mice, however an aberrant Th1/Th17 response may have also been induced.

4.2.5.3 Antihelminth effector molecules are intact following simultaneous inhibition of miR-99a-5p, miR-148a-3p and miR-155-5p

We next set out to investigate if previously described essential mechanisms of antihelminth were intact in miRNA-inhibited mice during 2^{o} *H. polygyrus*. Type 2 antihelminth immune mechanisms were unaffected by miRNA$^{3\Delta}$. Specifically, *H. polygyrus*-specific IgG1 production was unaffected by miRNA$^{3\Delta}$ (**Figure 4.11.A**) (McCoy et al., 2008). Intestinal expression of *Arg1*, a marker of aaMϕs and essential for immunity (Anthony et al., 2006), was also not significantly altered by miRNA$^{3\Delta}$, despite a trend to be increased compared to control inhibitor treatment (**Figure 4.11.B**). Similarly, the antihelminth molecule Relmβ *(Retnlb*), essential for immunity (Herbert et al., 2009), and *Ccl24*, a key chemokine in initiating protective immunity (Kannan et al., 2017), were also unaffected by miRNA$^{3\Delta}$ (**Figure 4.11.C-D**). Interestingly, expression of the endogenous anthelmintic PLA_2g1B (*Pla2g1b*), identified in **Chapter 3**, was decreased following miRNA$^{3\Delta}$, although not reaching statistical significance (**Figure 4.11.E**). Furthermore, the mucus response to 2^{o} challenge infection was also perturbed by miRNA$^{3\Delta}$, with the mucin genes *Muc1* and *Muc16* significantly decreased when compared to control inhibitor treated mice (**Figure 4.11.F-G**).

Overall, critical antihelminth effector molecule gene expression was intact following miRNA$^{3\Delta}$. However, the mucus response was significantly altered following miRNA$^{3\Delta}$

in 2º challenge infected mice. Whether a defective Muc1 and Muc16 response can perturb resistance to *H. polygyrus* is currently unclear, unlike Muc5ac which is required for expulsion of *T. muris* (Hasnain et al., 2011, Hasnain et al., 2010).

4.2.5.4 Increased antimicrobial signalling signature following simultaneous inhibition of miR-99a-5p, miR-148a-3p and miR-155-5p

With no obvious perturbation in type 2 immunity or critical downstream mediators following miRNA$^{3\Delta}$, we performed RNA sequencing of the small intestinal tissue 14 days-post 2º infection to identify the transcriptional impact of miRNA$^{3\Delta}$. We compared the intestinal transcriptome of mice following miRNA$^{3\Delta}$ to both control inhibitor treated mice and to vehicle treated mice, 14 days post 2º *H. polygyrus* infection This allowed us to confidently identify the transcriptional signature induced by concurrent inhibition of miR-99a-5p, miR-148a-3p and miR-155-5p and control for the impact of exogenous control miRNA inhibitor and vehicle treatment.

243 genes were identified to be significantly differentially expressed following miRNA$^{3\Delta}$ when compared to both the control inhibitor and vehicle treatment (p<0.05). We then analysed these 243 significantly differentially expressed genes using IPA® software. Upstream regulator analysis identified 13 molecules/factors which were predicted to be significantly activated following miRNA$^{3\Delta}$, relative to control inhibitor treatment. The majority of these factors are involved in the innate immune response to microbial pathogens, such as LPS, TNFα, TLR4, TICAM1, MyD88, CSF2 and IL-1β (**Figure 4.12.A**). Transcriptional changes responsible for the predicated activation of these upstream regulators are displayed in **Figure 4.12.B** (p<0.05, 1.5 fold change filter, relative to control inhibitor treatment). From this array of genes, we identified three critical antimicrobial genes, *Irg1* (also known as *Acod1*, Interferon response gene 1), *Tnfa* and *Lyz2* (lysozyme 2), all of which were upregulated following miRNA$^{3\Delta}$, relative to control inhibitor and vehicle treatment (**Figure 4.12.C-E**).

Overall, these data suggest that miRNAs miR-99a-5p, miR-148a-3p and miR-155-5p inhibit antimicrobial signalling pathways following 2º *H. polygyrus* challenge infection. Furthermore, inhibition of these miRNAs during 2º challenge infection

resulted in increased antimicrobial signalling, which has previously been associated with susceptibility to *H. polygyrus* (Reynolds et al., 2014a).

4.3 Discussion

miRNAs are a non-protein coding regulatory RNA species, transcribed from specific genes and function by regulating mRNA translation into protein (Ambros, 2003). This post-transcriptional fine-tuning function of gene expression has been implicated in a variety of biological settings, including immune responses. Never the less, the role of miRNAs in regulating immunity to intestinal helminth infection has not been well studied.

Intestinal helminth infections are highly prevalent in the developing world (Bethony et al., 2006, Hotez et al., 2008), with chronic infection associated with host morbidity (2016). Stalling vaccine efforts (Hotez et al., 2016) and the emergence of drug-resistant helminths (Albonico et al., 2003, Taman and Azab, 2014) calls for a greater understanding of antihelminth immunity to provide new therapeutic avenues.

In this chapter, we investigated the role of miRNAs in protective immunity to the intestinal helminth *H. polygyrus*. This model allowed us to compare susceptibility and resistance in the same host strain. We determined that a 50% global depletion of the miRNA processing enzyme Dicer, and subsequent depletion of mature miRNAs, did not influence resistance in C57BL/6 mice following a 1^{o} *H. polygyrus* infection. miRNA sequencing of the small intestine identified miRNAs that were differentially expressed in both susceptible and resistant mice following *H. polygyrus* infection. Specifically, we identified three miRNAs, miR-99a-5p, miR-148a-3p and miR-155-5p, that were uniquely upregulated in resistant mice. Individual pharmacological inhibition of each of these three miRNAs did not perturb resistance to 2^{o} *H. polygyrus* infection. However, combined inhibition of miR-99a-5p, miR-148a-3p and miR-155-5p abrogated resistance to 2^{o} *H. polygyrus* infection, likely through multiple mechanisms including dysregulated type 1 immune responses, antimicrobial responses and mucin gene expression

The remainder of this chapter discusses our methods and findings in the context of antihelminth immunity, highlighting implications, shortfalls and further questions.

4.3.1 The 'sledgehammer approach': Attempted global Dicer and miRNA knockdown ineffective

We hypothesised that miRNAs restrain protective responses to 1° *H. polygyrus* infection, permitting chronicity. Furthermore, we hypothesised that a global downregulation of miRNAs released a 'brake' on the immune system, permitting immune-mediated clearance of a 2° *H. polygyrus* infection. We tested this hypothesis by deleting Dicer, the miRNA processing enzyme, to downregulate miRNA expression prior to 1° *H. polygyrus* infection. We only achieved a 50% decrease in *Dicer* expression and a 50-60% reduction in miRNA expression (**Figure 4.1**). If we could achieve greater than 50-60% reduction in miRNA expression, we may be able to test our hypothesis. However, the genetic system we had used was imperfect and did not allow us to adequately test our hypothesis (**Figure 4.2**).

Reducing *Dicer*, and subsequent miRNA, expression by a maximum of 50% in the small intestine was a technical limitation of the mouse we generated, *R26*$^{CreERT2/yfp}$*Dicer*$^{fl/fl}$. Despite increased tamoxifen treatments, we were unable to reduce *Dicer* expression more than 50% (data not shown). Penetrance of tamoxifen and induction of Cre-recombinase may have been suboptimal in specific cells and/or tissues. It would be of interest to isolate different cell types from the small intestine following tamoxifen treatment to determine if *Dicer* expression was differentially reduced in different cell types, suggesting tamoxifen-induced Cre-recombinase activity was increased in those cells.

Furthermore, if the 50% reduction in *Dicer* expression was ubiquitous across all cells and tissues, the subsequent reduction of all functional miRNAs may mask the role of an essential miRNA, in a specific cell type, critical for antihelminth immunity. We therefore pursued a miRNA sequencing approach to identify specific miRNA species implicated in resistance to intestinal helminth infection.

miRNA sequencing of WT mice following 1° and 2° *H. polygyrus* infection confirmed differential expression of miRNAs in susceptible and resistant mice (**Figure 4.3**). However, a similar number of miRNAs were upregulated as downregulated in following 2° *H. polygyrus* infection, suggesting that miRNAs are not generally regulated, but are much more complex, suggesting that our initial hypothesis was incorrect.

4.3.2 miRNA sequencing

4.3.2.1 Issues, caveats and lessons learnt

Optimisation of miRNA sequencing used in this thesis posed a significant technical challenge. One particular area which required significant optimisation was the size separation of the small RNA library, to specifically enrich the sample for miRNAs. Automated methods were unsuccessful, resulting in a reduced miRNA read percentage. We overcame this by manually size selecting the miRNA fraction of the small RNA library using gel electrophoresis and manual extraction under UV illumination. This resulted in a significant increase in miRNA read percentage in 4 biological replicates for each group. To validate candidate miRNAs, any candidate miRNA identified from the miRNA sequencing was validated by qRT-PCR using the full 8 biological replicates.

An important limitation of our miRNA sequencing was the depth of sequencing. The number of reads produced was sub-optimum. A previous study demonstrated that sequencing depth of 12 million reads per sample allowed for the detection of approximately 80% of all expressed miRNAs, with lower depth significantly reducing the detection of miRNAs (Sun et al., 2014). In our data set we only achieved 1-3 million reads per sample and therefore were unlikely to detect lowly expressed miRNAs. As a result, our miRNA transcriptome analysis was likely to be incomplete, missing out on the differential expression of lowly expressed miRNAs.

Whole tissue miRNA sequencing also presented challenges when attempting to elucidate potential mechanisms of the differentially expressed miRNAs. The miRNA expression profile is likely to be different depending on cell type, with different miRNAs expressed in different cell types and specific miRNAs being more or less abundant in different cell types. Furthermore, the targets of a specific miRNAs can differ from cell type to cell type. Therefore, the action of the same miRNA may be different, for example, in a T cell to its action in a macrophage or epithelial cell. Consequently, despite miRNA sequencing of whole tissue giving us a broad view of the miRNA expression profile of the small intestine, we lacked resolution to identify in which cell type(s) the expression of the candidate miRNAs were critical. As a

result, elucidating a precise mechanism was challenging and further investigation is required in this area to both identify the cell type and the putative mRNA targets.

Overall, the miRNA sequencing performed in this Chapter was technically challenging and contained several potential limitations. However, early identification of these caveats enabled us to account for these as best as possible, particularly in the validation of candidate miRNA expression. We were therefore confident in our identification of the three candidate miRNAs, miR-99a-5p, miR-148a-3p and miR-155-5p, being differentially expressed in resistant mice. We also appreciate that, due to suboptimal sequencing depth, we may have missed miRNA species differentially expressed in our *H. polygyrus* infection model.

4.3.2.2 Intestinal miRNA profile changes following helminth infection

Despite the limitations of our miRNA sequencing data set, we were able to identify differentially expressed miRNAs in our model of susceptibility and resistance to *H. polygyrus* (**Figure 4.3**). Specifically, we identified 5 miRNAs that were differentially expressed 7 days-post 1^{o} infection (*H.p.* 1^{o}), with significantly more miRNAs differentially expressed 7 days-post 2^{o} infection (*H.p.* 2^{o}) (35 and 67 miRNAs on day 42 (*H.p.* 2^{o} (D42)) and 63 (*H.p.* 2^{o} (D63)) respectively (relative to naïve, $p<0.05$)). Interestingly, following drug-cure of 1^{o} infection and without 2^{o} infection (Rx (D42) and Rx (D63)), miRNAs were significantly differentially expressed in the small intestine and maintained until 49 days-post drug-treatment. The number of differentially expressed miRNAs in resistant mice, without 2^{o} infection, was less than that seen in resistant mice upon infection (16 and 19 miRNAs on day 42 (Rx (D42)) and 63 (Rx (D63)) respectively (relative to naïve, $p<0.05$)), suggesting that a suite of miRNAs was differentially expressed upon 2^{o} infection. Although not analysed in depth here, it would be interesting to study the miRNAs that are differentially expressed in the groups Rx (D42) and Rx (D63). This 'new baseline' of miRNA expression may confer protection against subsequent challenge infections.

Of the 5 miRNAs that were differentially expressed in *H.p.* 1^{o} (relative to naïve, $p<0.05$), it would be of interest to identify if their differential expression was required for susceptibility to *H. polygyrus* expression. Specifically, would pharmacological

manipulation of these miRNAs influence resistance to 1^{o} *H. polygyrus* infection? If this was indeed the case, understanding the mRNA targets of these miRNAs may uncover biological pathways that promote resistance to infection. Furthermore, do intestinal helminths induce changes in host miRNAs to promote chronic infection? Indeed, intestinal helminths have been demonstrated to produce immunomodulatory proteins and miRNAs which act on the host to promote susceptibility to infection (Maizels et al., 2012a, Grainger et al., 2010, Buck et al., 2014). However, whether these products alter host miRNA expression is unclear.

4.3.3 Individual miRNA inhibition

4.3.3.1 Why was individual miRNA inhibition ineffective?

Following the observation that miRNAs miR-99a-5p, miR-148a-3p and miR-155-5p were upregulated in mice resistant to *H. polygyrus* (**Figure 4.4**), we tested whether these miRNAs were functionally important for protective immunity to *H. polygyrus* infection. Despite significant inhibition of the miRNAs, using pharmacological inhibition of each miRNA individually during 2^{o} challenge infection did not impact resistance. We therefore concluded that miR-99a-5p, miR-148a-3p and miR-155-5p were not essential for resistance to *H. polygyrus*. However, a previous study illustrated that genetic deletion of miR-155 abrogated immunity to 2^{o} *H. polygyrus* infection (Okoye et al., 2014), suggesting miR-155 is essential for resistance to *H. polygyrus*. In light of this discrepancy, it would be of interest to create both miR-99a-5p$^{-/-}$ and miR-148a-3p$^{-/-}$ mice and assess whether genetic deletion of these miRNAs will also abrogate immunity to *H. polygyrus* infection. The importance in understanding the differences between transient pharmacological inhibition and germline genetic deletion studies is discussed below in section **4.3.3.2**.

One potential explanation of why the miRNA inhibitors did not abrogate resistance to *H. polygyrus* is that they did not effectively inhibit miRNA expression and function in all target tissues and cells. This is however unlikely. The miRNA inhibitors used in these experiments function by competitively binding to their target miRNA, preventing the miRNA binding to mRNA. Therefore, the miRNA inhibitors function by blocking miRNA binding, rather than by directly downregulating miRNA expression.

We determined that the miRNA inhibitors decreased the target miRNA expression by at least 85%, with the remaining miRNAs likely not functional due to binding by the miRNA inhibitors, although this has not been demonstrated. To confirm the efficacy of the miRNA inhibitors, the expression of the mRNA targets must be compared to both control inhibitor treatment and the miRNA knockout mouse.

A second explanation of why the miRNA inhibitors did not abrogate resistance to *H. polygyrus* is that the timing of inhibition within the infection model was not optimal. This is especially imperative for both miR-99a-5p and miR-148a-3p as they were both upregulated in resistant mice, without 2^{o} infection (**Figure 4.4**). Perhaps, the critical functions of both miR-99a-5p and miR-148a-3p in mediating resistance are immediately after drug-treatment and not during 2^{o} challenge infection. If this were to be the case the transient knockdown of these two miRNAs during 2^{o} infection would not abrogate resistance. Therefore, treating the mice during drug treatment and throughout 2^{o} infection maybe more appropriate.

Another possible explanation as to why the individual inhibition of the miRNAs did not abrogate resistance to *H. polygyrus* is that miR-99a-5p, miR-148a-3p and miR-155-5p cross-regulate the same pathway(s) and perturbation of an individual miRNA is not enough to abrogate these pathways. Instead, all three candidate miRNAs would need to be concurrently inhibited to release the pathway(s) from miRNA-regulation and abrogate resistance to *H. polygyrus*. This is discussed further in section **4.3.4**.

4.3.3.2 Pharmacological inhibitors versus knockout mice: lessons for potential therapeutics

In section **4.2.4**, we determined that individual inhibition of the three candidate miRNAs, miR-99a-5p, miR-148a-3p and miR-155-5p, did not perturb protective immunity to 2^{o} *H. polygyrus* infection, despite significantly reducing miRNA expression by at least 85%. Conversely, a previous study demonstrated that miR-155$^{-/-}$ mice failed to expel a 2^{o} *H. polygyrus* challenge infection (Okoye et al., 2014). The discrepancy between the results of these two independent studies may highlight

the differences between pharmacological inhibition and genetic deletion models. A head-to-head comparison would be required to confirm this discrepancy.

The key difference between genetic deletion and pharmacological inhibition is the constitutive nature of the deletion in the knockout mouse versus the temporal downregulation following inhibitor administration. In light of this, both the timing and complete removal of the miRNA is likely to be important, as discussed above in section **4.3.3.1**. This is of great importance when considering the translation of results from genetic deletion studies for potential use in the clinic. Pharmacological inhibitor treatment may not reproduce the effects seen following genetic deletion and it is therefore essential to validate findings using models of genetic deletion with pharmacological inhibition. Investigating the differences observed using these two approaches may further improve our understanding of the biology in question, specifically in the thresholds of gene/protein expression, protein activity versus scaffold function and the design of pharmacological agents.

In the context of miRNAs, these findings highlight a fundamental importance of validating results identified using miRNA-knockout mice with pharmacological inhibitors. Many conclusions have been drawn using specific miRNA-knockout mice in the context of *in vivo* disease modelling. Whether pharmacological inhibition of these specific miRNAs would replicate the phenotype observed in knockout mice remains undetermined in the majority of cases.

4.3.4 The power of three: Concurrent miRNA inhibition abrogated protective immunity

Despite individual inhibition of the three candidate miRNAs, miR-99a-5p, miR-148a-3p and miR-155-5p, being ineffective in perturbing protection against *H. polygyrus*, we identified that simultaneous inhibition of the three candidate miRNAs prevented expulsion of a 2^{o} *H. polygyrus* challenge infection (**Figure 4.9**). However, further studies are required to confirm this finding and identify potential mechanisms.

4.3.4.1 Do miRNAs miR-99a-5p, miR-148a-3p and miR-155-5p cooperate to regulate intestinal immune homeostasis?

Analysis of the T cell response following combined miRNA inhibition revealed a reduced frequency of type 2 cytokine-producing T cells and an increased frequency of type 1 and type 17 T cells in the spleen. However, this was not observed in the local draining mLNs (see section **4.2.5.2**). It would be of interest to further analyse the T cell response 5-7 days-post infection, rather than at day 14, to determine if miRNA inhibition perturbed Th2 immunity at the peak of the memory response. Moreover, analysing T cell cytokine production in the small intestine during 2^{o} infection would also allow us to determine if the T cell response was intact at the site of infection. However, this is a limitation of the infection model as the recovery of live cells from the intestine following 2^{o} *H. polygyrus* infection is impossible.

Analysis of the small intestine determined that the expression of critical antihelminth effector molecules Arginase 1 and Relmβ were intact following triple miRNA inhibition (Anthony et al., 2006, Herbert et al., 2009). Furthermore, the gene expression of Ccl24, a chemokine demonstrated to be important in promoting resistance to *H. polygyrus* (Kannan et al., 2017), was also intact. Interestingly, the expression of the endogenous anthelmintic PLA_2g1B was seen to be decreased as a result of triple miRNA inhibition, although not reaching statistical significance. In **Chapter 3**, we demonstrated that intestinal *Pla2g1b* expression was dependent upon the microbiota and functional immune cells. Whether IEC *Pla2g1b* expression is directly or indirectly regulated by miR-99a-5p, miR-148a-3p and/or miR-155-5p is unclear, although *Pla2g1b* is not a predicted mRNA target of miR-99a-5p, miR-148a-3p or miR-155-5p. We also observed that concurrent inhibition of these three miRNAs prevented the upregulation of the cell surface mucin genes *Muc1* and *Muc16*. The gel-forming mucins Muc2 and Muc5ac are essential for the expulsion of *T. muris* (Hasnain et al., 2011, Hasnain et al., 2010), whereas intestinal *Muc1* expression correlated with expulsion of *N. brasiliensis* (Turner et al., 2013). Muc1 has previously been demonstrated to be a critical intestinal barrier function, enhancing protection against bacteria and bacterial toxins (McAuley et al., 2007). Both Muc 1 and Muc16 have also been implicated in mucosal homeostasis, with both mucins demonstrated to supress TLR signalling in epithelial cells perhaps through the formation of a physical

barrier preventing epithelial cell contact with TLR agonists (Menon et al., 2015). The roles of Muc1 or Muc16 have not been addressed in antihelminth immunity. Perhaps these two mucins act to disrupt the intestinal niche of *H. polygyrus*, forming part of the 'weep and sweep' response. Alternatively, the upregulation of these two cell surface mucins could provide protection against the translocation of the intestinal microbiota into the intestinal tissue, following damage elicited from intestinal helminth infection, reduce antimicrobial signalling and promote protective immunity to *H. polygyrus* (Reynolds et al., 2014a). This hypothesis is outlined in **Figure 4.13.A**.

Transcriptional analysis of the small intestine suggested that the concurrent inhibition of miR-99a-5p, miR-148a-3p and miR-155-5p led to increased antimicrobial signalling, increasing the expression of antimicrobial response genes, including *Irg1*, *Tnfα* and *Lyz2*, and a predicted increase in activation of antimicrobial molecules, such as LPS, TLR4, MyD88 and TNFα (see section **4.2.5.4**). MyD88 is an essential molecule in the signal transduction of IL-1 and TLR signalling pathways (Medzhitov et al., 1998, Adachi et al., 1998). MyD88 signalling has previously been demonstrated to inhibit protective immunity to *H. polygyrus* infection (Reynolds et al., 2014a), suggesting that microbial detection and antimicrobial responses promote susceptibility. Therefore, the abrogation of protective immunity and worm expulsion, induced by simultaneous inhibition of miR-99a-5p, miR-148a-3p and miR-155-5p following 2^{o} *H. polygyrus* challenge infection, maybe due to increased MyD88-mediated antimicrobial signalling. An increased antimicrobial response may also be responsible for the increased Th1 and Th17 cytokine response identified in the spleen, with both Th cell subsets important in the resolution of bacterial and fungal infections (Luckheeram et al., 2012). We therefore hypothesise that miR-99a-5p, miR-148a-3p and miR-155-5p inhibit TLR-MyD88-mediated antimicrobial signalling, which allows for protective immunity and expulsion of 2^{o} *H. polygyrus* infection (**Figure 4.13.B**). Whether these miRNAs directly or directly regulate antimicrobial signalling pathways and target gene expression remains unclear in our model. Previous studies have demonstrated that miR-99a-5p, miR-148a-3p and miR-155-5p can directly modulate MyD88-NF-ϰB signalling pathways and response genes (see section **4.3.4.2** below).

In conclusion of these data, we hypothesise that in resistant mice, the upregulation of miRNAs miR-99a-5p, miR-148a-3p and miR-155-5p either function to maintain

barrier function and contribute to antimicrobial responses (**Figure 4.13.A**), indirectly explaining the elevated anti-microbial responses seen upon miRNA inhibition, or directly inhibit excessive antimicrobial signalling (**Figure 4.13.B**) allowing protective immunity to be unimpeded. Future work is required to confirm, test and validate these initial findings and hypotheses of how these miRNAs contribute to resistance to intestinal helminth infection

4.3.4.2 Regulation of antimicrobial signalling by miR-99a-5p, miR-148a-3p and miR-155-5p and impact on immunity to intestinal helminth infection.

The activation of TLRs and subsequent antimicrobial responses can be regulated by miR-99a-5p, miR-148a-3p and miR-155-5p. Specifically, miR-148a directly targets DNA methyltransferase (DNMT)1 causing the hypomethylation and upregulation of SOCS1 which in turn directly inhibits TLR3 and TLR4 signalling in DCs (Liu et al., 2016). miR-99a overexpression attenuates LPS-induced apoptosis and miR-99a inhibition potentiated LPS-induced apoptosis activity in cardiomyocytes (Jing et al., 2017). In contrast to miR-99a and miR-148a, miR-155 has been demonstrated to positively regulate LPS-induced TLR4 signalling, with miR-155 expression upregulated following LPS stimulation of macrophages and required for sustaining LPS-induced TNFα responses (Cheng et al., 2012, Mantuano et al., 2016, Li et al., 2013). Interestingly, several helminth products can to bind to TLRs, such as double-stranded RNA and the lipid lysophosphatidylserine from *Schistosoma mansoni*, which binds to TLR3 and TLR2, respectively (Aksoy et al., 2005, van der Kleij et al., 2002). Similarly, the ES product ES-62 from the filarial nematode *Acanthocheilonema viteae* stimulates TLR4 (Goodridge et al., 2005, Goodridge et al., 2004). *H. polygyrus* infection is also likely to result in host TLR signalling not only via ES products, but through exposure to the microbiota following physical disruption of the epithelial barrier upon infection. We can therefore postulate that these microbial signals are likely to impair the hosts ability to induce a protective type 2 immune response upon infection. Indeed, *Myd88*-deficient mice have an increased protective immune response to 1^{o} *H. polygyrus* infection (Reynolds et al., 2014a).

The NF-κB signalling cascade is one component of the MyD88-mediated antimicrobial signalling pathway which initiates the transcription of a variety of antimicrobial and inflammatory response genes (Medzhitov et al., 1998, Adachi et al., 1998). miRNAs miR-99a-5p, miR-148a-3p and miR-155-5p can regulate NF-κB signalling molecules and target genes. Specifically, miR-99a can inhibit LPS-induced NF-κB translocation to the nucleus and target gene transcription, such as TNFα, IL-1β and IL-6 in endothelial cells (Bao et al., 2016). Furthermore, both miR-148a and miR-155 also directly target RelA, an essential component of NF-κB signalling, thus inhibiting NF-κB signal transduction (Bao and Lin, 2014). In addition, miR-148a-3p also directly targets IκB kinase (IKK)-β, the catalytic subunit responsible for NF-κB activation (Ghosh and Karin, 2002), also preventing NF-κB translocation to the nucleus and target gene transcription (Patel et al., 2015). The role of NF-κB signalling has been investigated in immunity to intestinal helminth infection. Both NF-κB1- and NF-κB2-deficient mice failed to expel *T. muris* infection, with both strains inducing an inappropriate Th1 response (Artis et al., 2002). A later study demonstrated that IKK-β expression in IECs was required for expulsion of *T. muris*, specifically in inducing TSLP expression and subsequent initiation of Th2 cell-dependent-immunity (Zaph et al., 2007). Whether NF-κB signalling is required for immunity to *H. polygyrus* remains unclear.

We hypothesised that the upregulation of miRNAs miR-99a-5p, miR-148a-3p and miR-155-5p in resistant mice regulates antimicrobial immunity or signalling, indirectly influencing protective immunity to *H. polygyrus*. We can test whether the miRNA-regulation of antimicrobial signalling is required for protective immunity to *H. polygyrus*. To do this we could treat *Myd88*-deficient mice with combined administration of miR-99a-5p, miR-148a-3p and miR-155-5p inhibitors in an attempt to rescue protective immunity. If these miRNA inhibitor-treated mice expel a 2^{o} *H. polygyrus* infection we can conclude that the upregulation of miRNAs miR-99a-5p, miR-148a-3p and miR-155-5p in resistant mice is essential for immunity to *H. polygyrus* through the regulation of antimicrobial signalling.

Taken together, these data support our hypothesis and indicate that miR-99a-5p, miR-148a-3p and miR-155-5p may cooperate to inhibit various aspects of antimicrobial responses, directly or indirectly, allowing for unimpeded protective immunity and expulsion of 2^{o} *H. polygyrus* challenge infection. Furthermore, the co-

regulation of a specific pathway by these three independent miRNAs may explain why inhibition of one single miRNA was ineffective in perturbing immunity to *H. polygyrus*; as the three miRNAs may have some level of redundancy in effectively suppressing these antimicrobial pathways.

4.3.5 The importance of miRNAs in antihelminth immunity

Overall, the experiments presented in this Chapter demonstrate that miRNAs are differentially regulated following intestinal helminth infection, with distinct miRNA profiles in mice with acquired resistance to *H. polygyrus* compared to susceptible mice. Furthermore, we identified three miRNAs, miR-99a-5p, miR-148a-3p and miR-155-5p, that were upregulated in resistant mice during 2^{o} *H. polygyrus* infection and were essential for resistance to 2^{o} *H. polygyrus* infection. Taken together, these data illustrate an essential role of miRNAs in regulating functional antihelminth immunity and highlight an understudied area of research in immunity to intestinal helminth infections.

Further research is required to elucidate the proposed coordinated mechanisms of miR-99a-5p, miR-148a-3p and miR-155-5p in regulating immunity to *H. polygyrus*. Extensive research should be focussed in delineating the role of specific miRNAs in cell types critical for- and in the context of immunity to intestinal helminth infections. This is crucial to overcome the limitation of whole tissue miRNA sequencing, especially with the same miRNA being expressed in multiple cell types with the potential of mediating different functions. Currently, the miRNA repertoire and function has only been studied succinctly in T cells, within antihelminth immunity (Okoye et al., 2014, Kelada et al., 2013).

A potentially exciting area of investigation is the role of miRNAs in host-pathogen interactions. Recent studies have elegantly illustrated the transfer of *H. polygyrus*-derived miRNAs to the host as a mechanism to downregulate antihelminth immune responses (Buck et al., 2014). Whether the opposite is also true, with host-derived miRNAs transferred to helminths is unclear. For example, it is interesting to speculate that host-derived miRNAs, upregulated in resistant mice, are released, transferred

and taken up into intestinal helminths to impair parasite health through miRNA-mRNA target inhibition, leading to expulsion.

A greater understanding the of miRNAs in immunity to intestinal helminth infections may uncover novel antihelminth biological pathways and improve our understanding of antihelminth immunity.

4.4 Figures

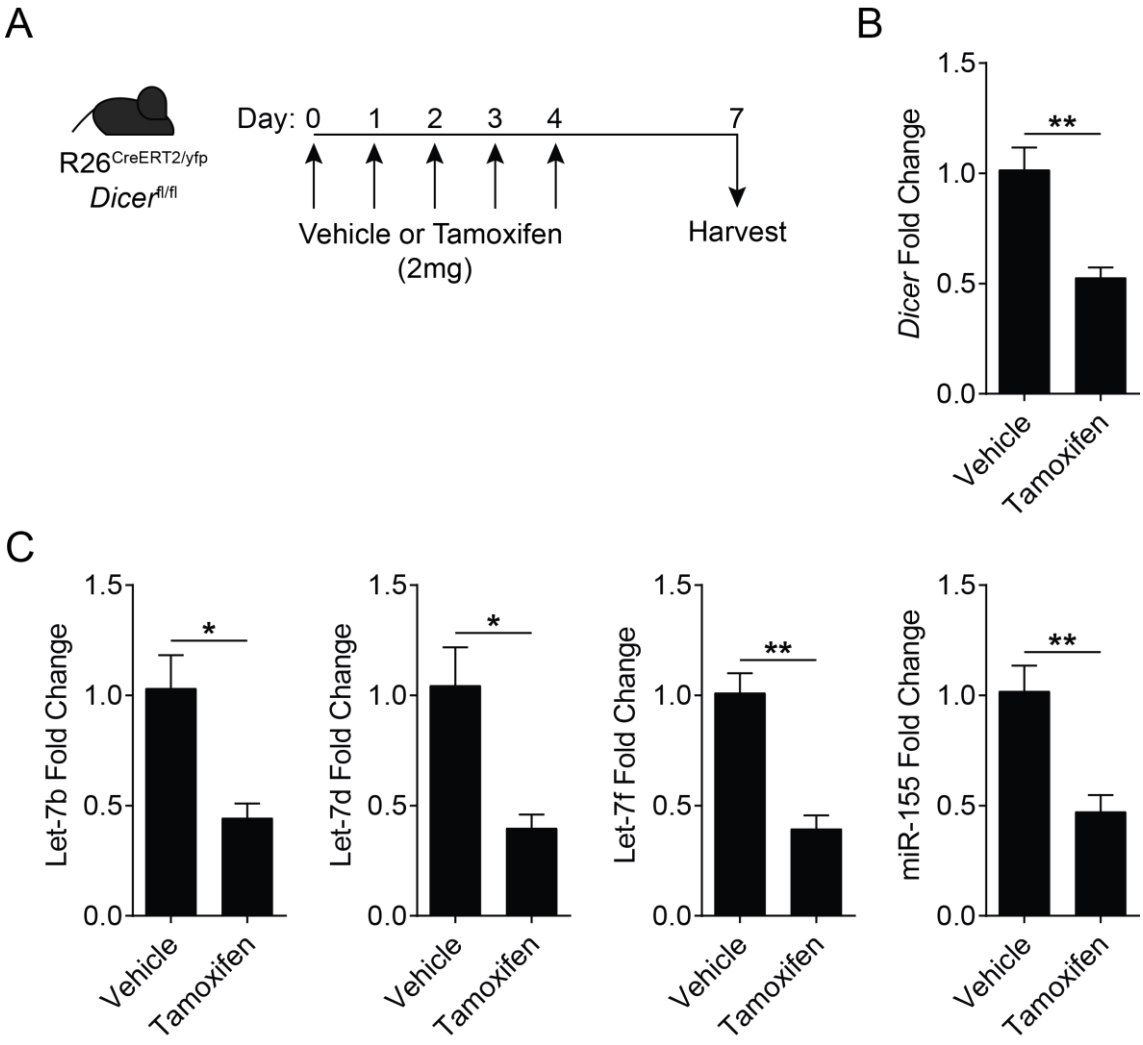


Figure 4.1 Inducible *Dicer* deletion perturbs mature miRNA biogenesis in the small intestine

(**A**) *R26*$^{CreERT2/yfp}$*Dicer*$^{fl/fl}$ mice were treated with 2mg of tamoxifen or vehicle control on 5 consecutive days (1mg *i.p.* and 1mg oral gavage) and the small intestine was harvested 3 days after the final treatment (Day 7). (**B**) *Dicer* expression in the duodenal tissue at day 7. (**C**) miRNA species Let-7b, Let-7d, Let-7f and miR-155 expression in the duodenal tissue at day 7. Data represented as mean ± SEM, n=4. All data is representative of at least two independent experiments. * = p<0.05, ** = p<0.01 determined using an unpaired two-tailed t test

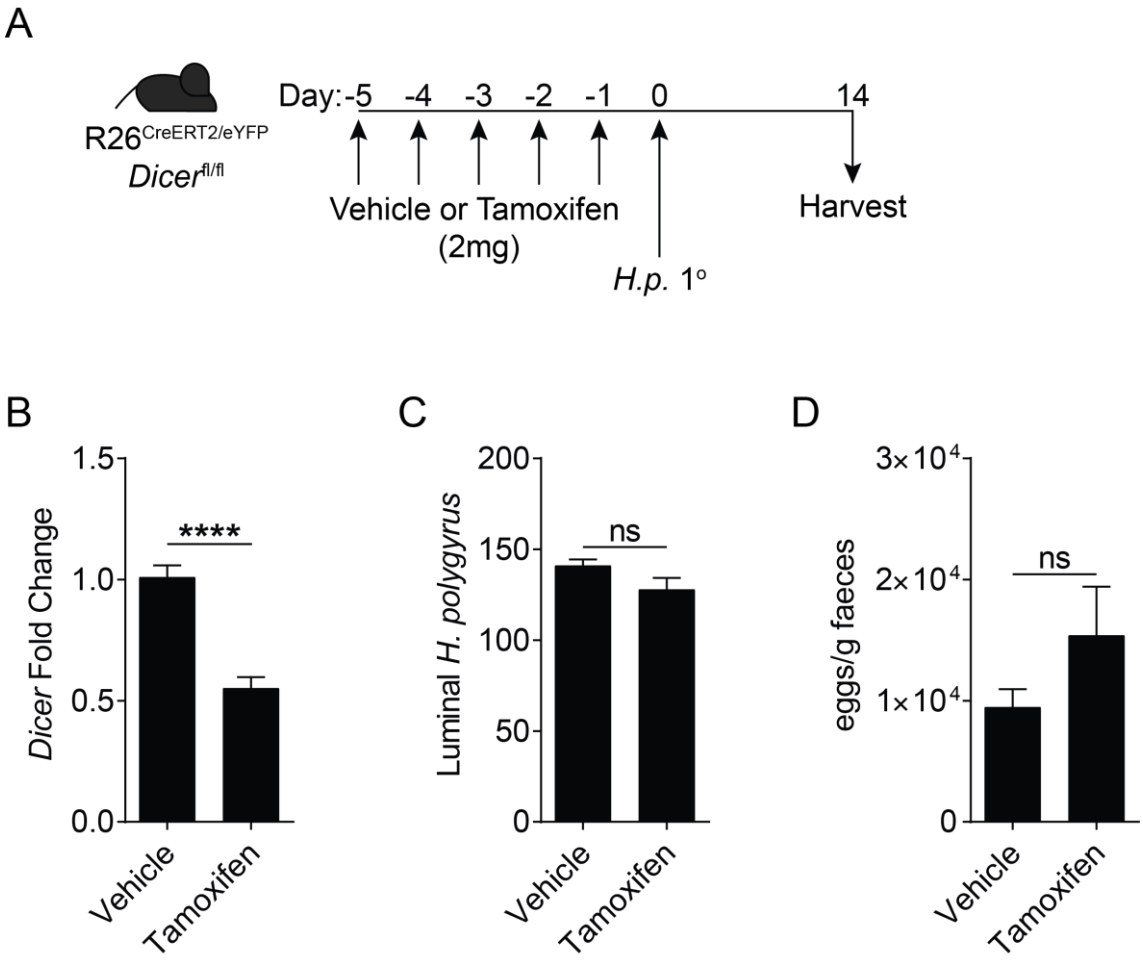


Figure 4.2 Inducible *Dicer* deletion in the small intestine does not promote resistance to *H. polygyrus*

(**A**) *R26*$^{CreERT2/yfp}$*Dicer*$^{fl/fl}$ mice were treated with 2mg of tamoxifen or vehicle control on 5 consecutive days from day -5 to -1 (1mg *i.p.* and 1mg oral gavage). Mice were then orally infected with 200 L3 *H. polygyrus* larvae on day 0 and the small intestine was harvested 14 days-post infection. (**B**) *Dicer* expression in the duodenal tissue at day 14. (**C**) Luminal *H. polygyrus* worms in the small intestine 14 days-post infection. (**D**) Faecal egg counts 14 days-post *H. polygyrus* infection. Data represented as mean ± SEM, n=8-9. All data is representative of at least two independent experiments. ns = not significant, **** = $p<$ 0.0001 determined using an unpaired two-tailed t test

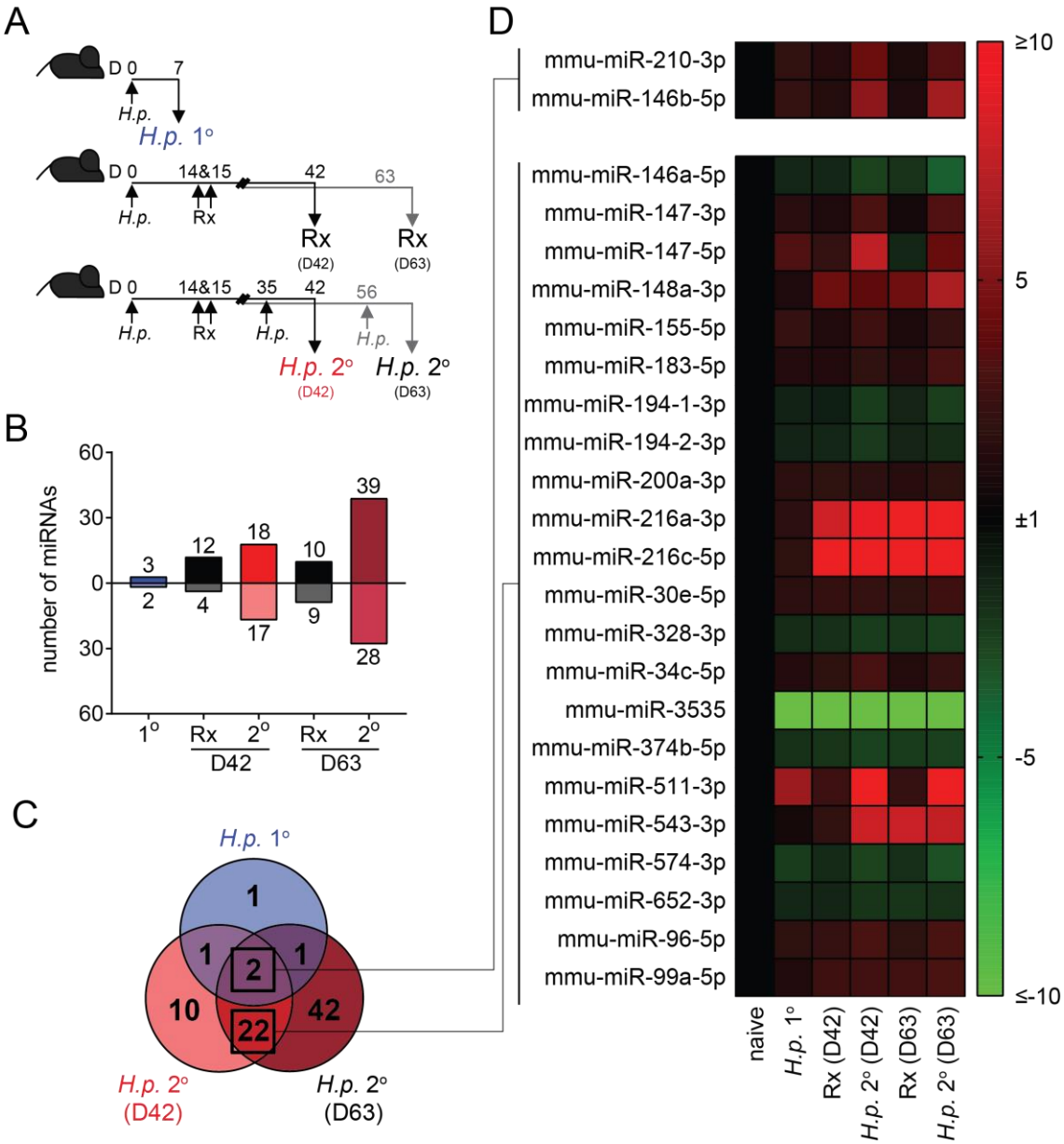


Figure 4.3 miRNA sequencing of intestinal tissue identified miRNAs associated with resistance to *H. polygyrus*

(**A**) C57BL/6 mice were orally infected with 200 L3 *H. polygyrus* larvae on day 0. A cohort of mice was sacrificed 7 days-post 1º *H. polygyrus* infection (*H.p.* 1º). Remaining mice were drug treated (Rx) on days 14 and 15. Two cohorts of mice were then harvest on day 42 or Day 63 (Rx (D42) and Rx (D63), respectively). Another two cohorts of mice were then 2º challenge infected with *H. polygyrus* on day 35 or day 56 and harvested 7 days-post 2º infection (*H.p.* 2º (D42) and *H.p.* 2º (D63), respectively). RNA was harvested from the duodenum at each time point. (**B**) The number of miRNAs significantly differentially expressed in *H.p.* 1º, Rx (D42), Rx (D63), *H.p.*2º (D42) and *H.p.*2º (D63) (up- and downregulated, relative to naïve, $p<0.05$). (**C**) Common and differentially expressed miRNAs in *H.p.* 1º, *H.p.*2º (D42) and *H.p.*2º (D63) (relative to naïve, $p<0.05$). (**D**) Expression profile of candidate miRNAs implicated in resistant to *H. polygyrus* identified in (**C**). n=4.

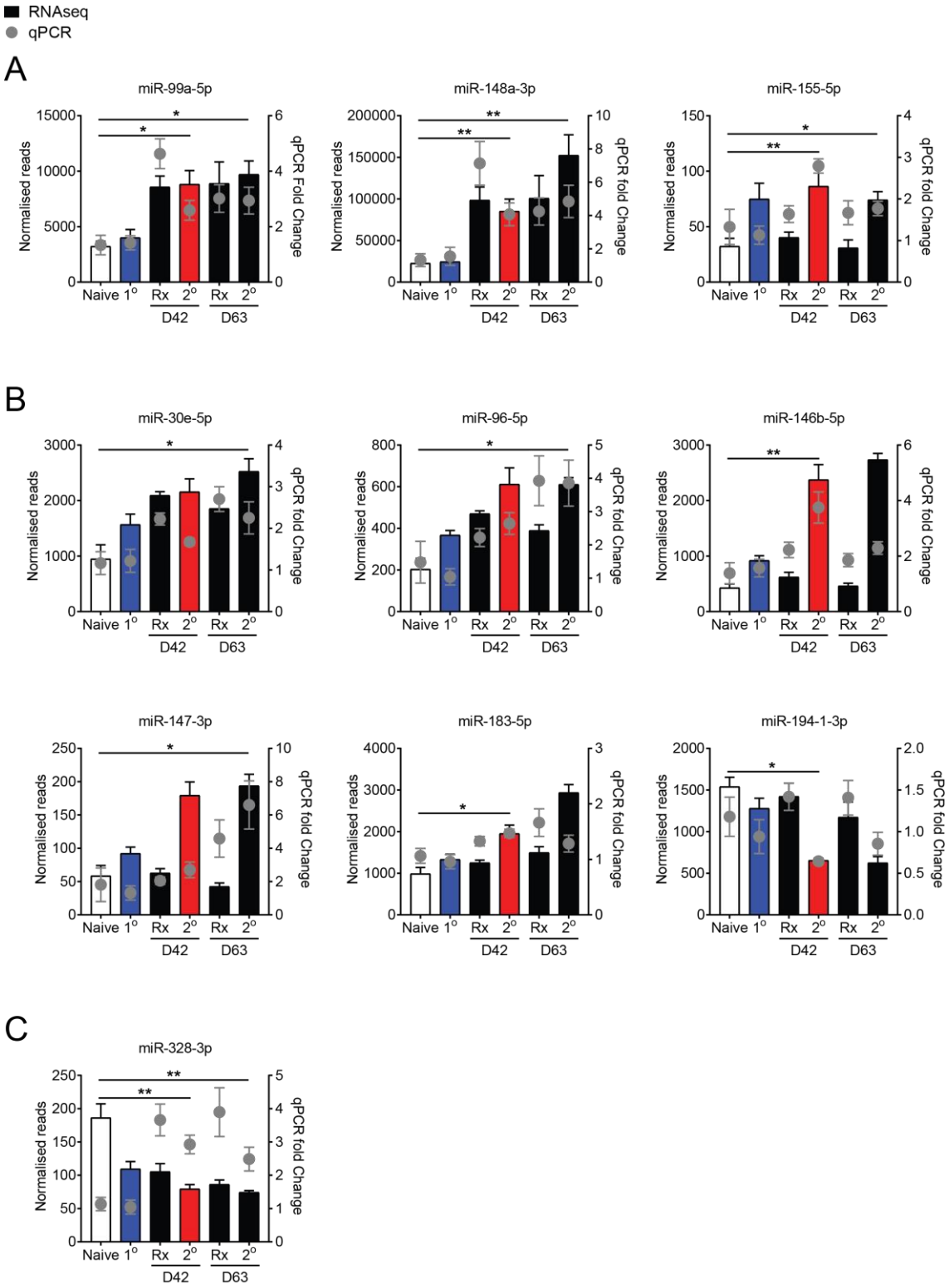

RNAseq
qPCR
A
miR-99a-5p
miR-148a-3p
miR-155-5p
B
miR-30e-5p
miR-96-5p
miR-146b-5p
miR-147-3p
miR-183-5p
miR-194-1-3p
C
miR-328-3p
Normalised reads
qPCR fold Change
Naive 1°
Rx
2°
D42
D63

Figure 4.4 qRT-PCR validation of candidate miRNAs implicated in resistance to *H. polygyrus*

(**A**) miR-99a-5p, miR-148a-3p and miR-155-5p; (**B**) miR-30-5p, miR-96-5p; miR-146b-5p, miR-47-3p, miR-183-5p and miR194-1-3p; (**C**) miR-328-3p expression in small intestine from RNA sequencing data, n=4. Significance confirmed by qPCR, n=8. Data represented as mean ± SEM. * = $p<0.05$, ** = $p< 0.01$ determined using a one-way ANOVA with Dunnett's multiple comparison analysis.

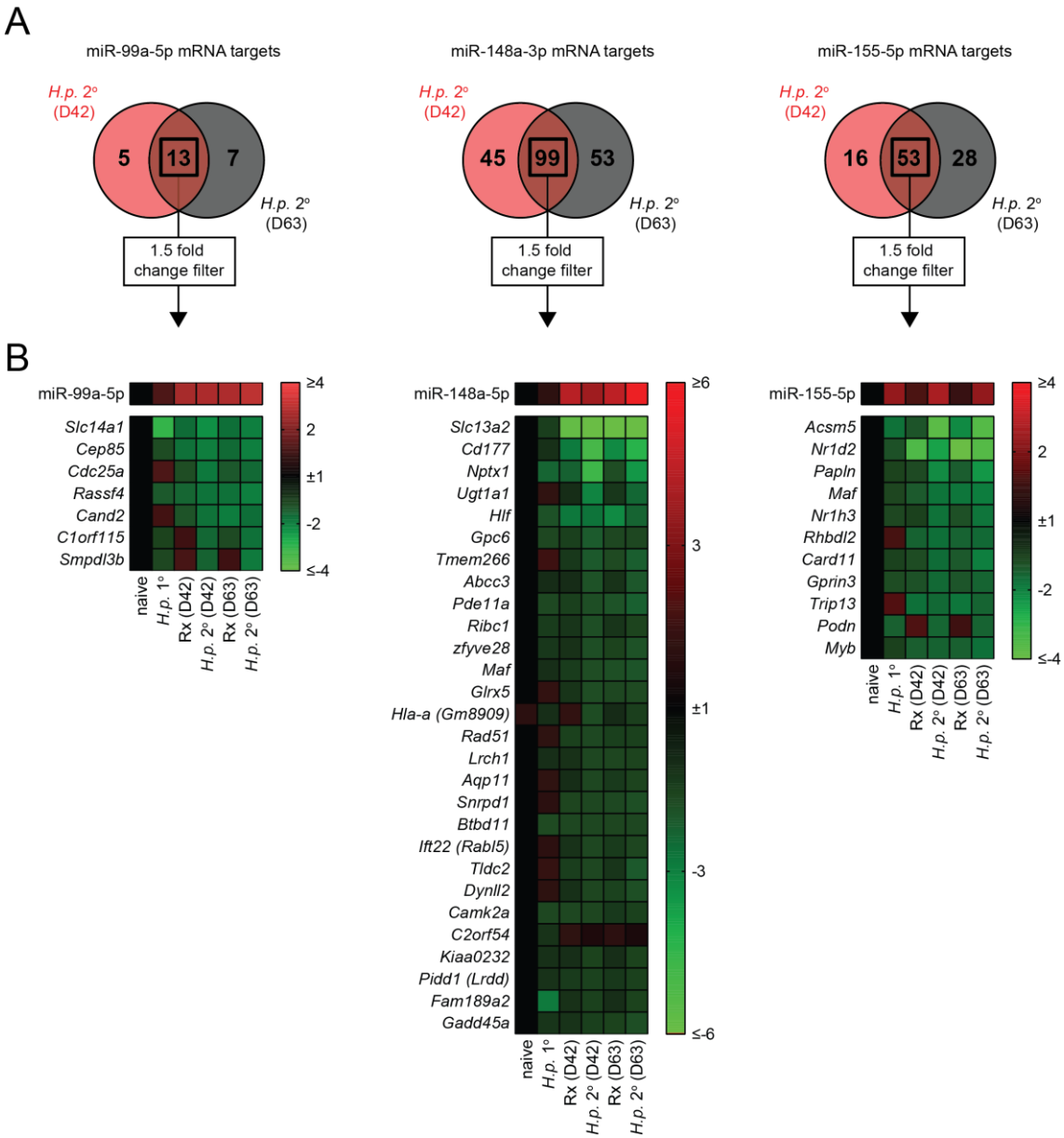


Figure 4.5 Complementary mRNA sequencing and *in silico* analysis identified candidate miRNA putative mRNA targets

(**A**) IPA analysis and identification of miR-99a-5p, miR-148a-3p and miR-155-5p expression-paired putative mRNA targets significantly expressed in *H.p.*2º (D42) and *H.p.*2º (D63) (relative to naïve, $p<0.05$). Data from mRNA sequencing (shown in **Figure 3.1**) complementary to miRNA sequencing (shown in **Figure 4.3**). (**B**) Expression profile of candidate miRNA and expression-paired putative mRNA targets identified in (**A**) (1.5 fold change filter, relative to naïve, $p<0.05$).

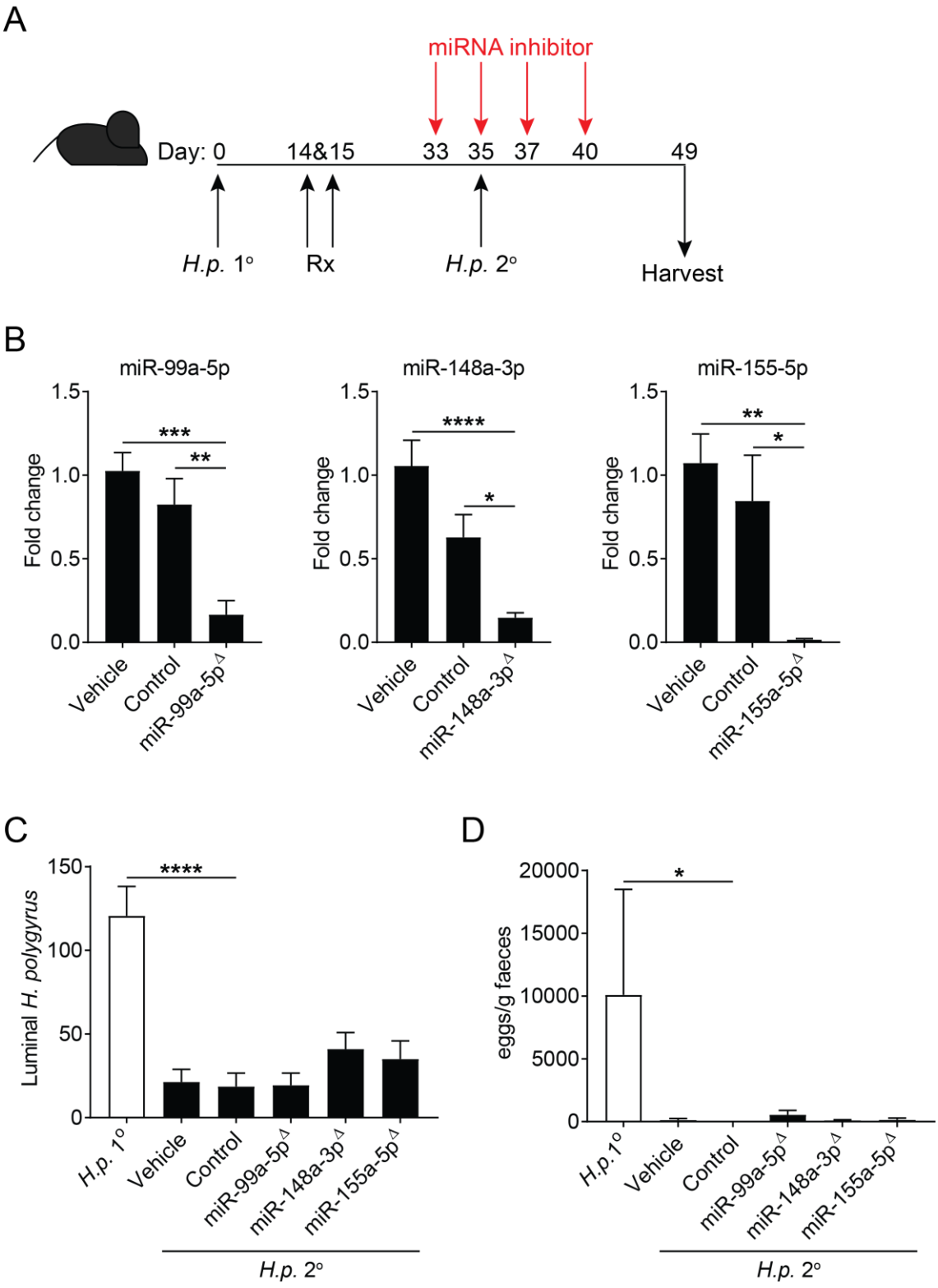

A
miRNA inhibitor
Day: 0
14&15
33
35
37
40
49
H.p. 1°
Rx
H.p. 2°
Harvest
B
miR-99a-5p
miR-148a-3p
miR-155-5p
Fold change
1.5
1.0
0.5
0.0
***
**
****
*
Vehicle
Control
miR-99a-5p^Δ
miR-148a-3p^Δ
miR-155a-5p^Δ
C
Luminal H. polygyrus
150
100
50
0
****
H.p. 1°
Vehicle
Control
miR-99a-5p^Δ
miR-148a-3p^Δ
miR-155a-5p^Δ
H.p. 2°
D
eggs/g faeces
20000
15000
10000
5000
0
*
H.p.1°
Vehicle
Control
miR-99a-5p^Δ
miR-148a-3p^Δ
miR-155a-5p^Δ
H.p. 2°

Figure 4.6 Individual candidate miRNA inhibition does not abrogate resistance to 2^{o} *H. polygyrus* infection

(**A**) C57BL/6 mice were orally infected with 200 L3 *H. polygyrus* larvae on day 0 (*H.p.* 1^{o}). Mice were then drug treated (Rx) on days 14 and 15 and 2^{o} challenge infected on day 35 (*H.p.* 2^{o}). Mice were treated with miRNA inhibitors, negative control inhibitor or vehicle only on days 33, 35, 37 and 40. Mice were then sacrificed on day 49 (14 days-post 2^{o} infection). (**B**) miR-99a-5p, miR-148a-5p and miR-55-5p expression in the small intestine following treatment with miRNA inhibitor (miR-99a-5p$^{\Delta}$, miR-148a-5p$^{\Delta}$ or miR-55-5p$^{\Delta}$), negative control inhibitor (control) or vehicle only. (**C**) Luminal *H. polygyrus* adult worms in the small intestine and (**D**) faecal egg burden on day 49 following treatment with miRNA inhibitor (miR-99a-5p$^{\Delta}$, miR-148a-5p$^{\Delta}$ or miR-55-5p$^{\Delta}$), negative control inhibitor (control) or vehicle only. Data represented as mean ± SEM, n=5. All data is representative of two independent experiments. * = p<0.05, ** = p< 0.01, *** = p< 0.001, **** = p< 0.0001 determined using a one-way ANOVA with Dunnett's multiple comparison analysis.

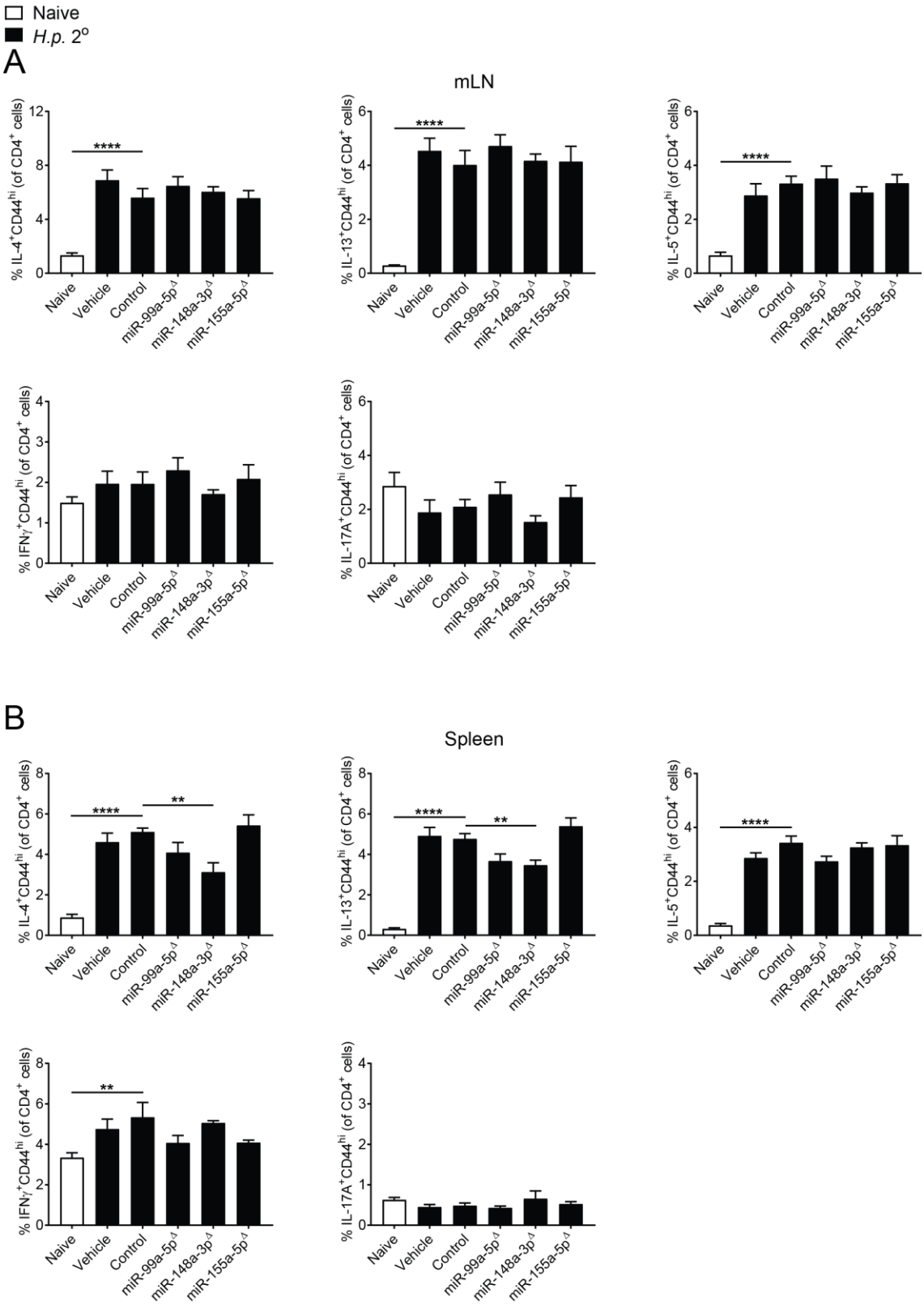

Naive
H.p. 2°
A
mLN
% IL-4+CD44hi (of CD4+ cells)
% IL-13+CD44hi (of CD4+ cells)
% IL-5+CD44hi (of CD4+ cells)
% IFNγ+CD44hi (of CD4+ cells)
% IL-17A+CD44hi (of CD4+ cells)
****
Naive
Vehicle
Control
miR-99a-5p4
miR-148a-3p4
miR-155a-5p4
B
Spleen
**

Figure 4.7 Individual miRNA inhibition does not alter T cell response following 2º *H. polygyrus* infection

The frequency of cytokine producing $CD44^{hi}$ $CD4^{+}$ T cells in naive mice or 14 days-post 2^{o} *H. polygyrus* infection following treatment with vehicle only, negative control inhibitor (control) or miRNA inhibitor (miR-99a-5p$^{\Delta}$, miR-148a-5p$^{\Delta}$ or miR-55-5p$^{\Delta}$) in the mLN (**A**) and spleen (**B**). Data represented as mean ± SEM, n=5. All data is representative of two independent experiments. ** = $p< 0.01$, **** = $p< 0.0001$ determined using a one-way ANOVA with Dunnett's multiple comparison analysis.

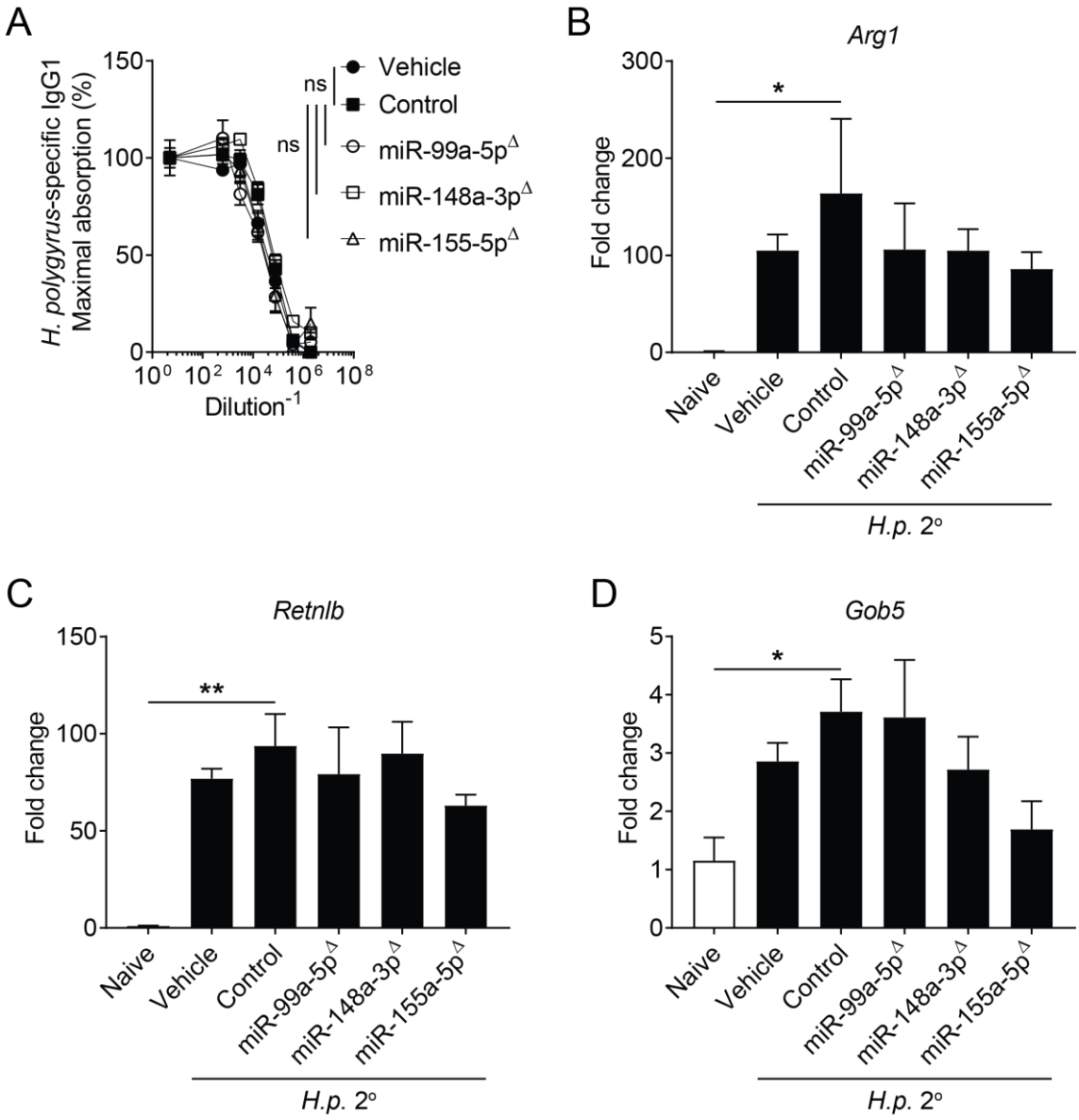


Figure 4.8 Antihelminth effector responses are intact following individual miRNA inhibition

(**A**) *H.* polygyrus-specific IgG1 in the serum from mice 14 days-post 2° *H.* polygyrus infection following treatment with vehicle only, negative control inhibitor (control) or miRNA inhibitor (miR-99a-5p$^{\Delta}$, miR-148a-5p$^{\Delta}$ or miR-55-5p$^{\Delta}$). Expression of *Arg1* (**B**), *Retnlb* (**C**) and *Gob5* (**D**) in the small intestine 14 days-post 2° *H.* polygyrus infection following treatment with vehicle only, negative control inhibitor (control) or miRNA inhibitor (miR-99a-5p$^{\Delta}$, miR-148a-5p$^{\Delta}$ or miR-55-5p$^{\Delta}$), relative to naïve mice. Data represented as mean ± SEM, n=5. All data is representative of two independent experiments. ns = not significant, * = p<0.05, ** = p< 0.01 determined using two-way ANOVA withDunnett's multiple comparison analysis or a one-way ANOVA with Dunnett's multiple comparison analysis.

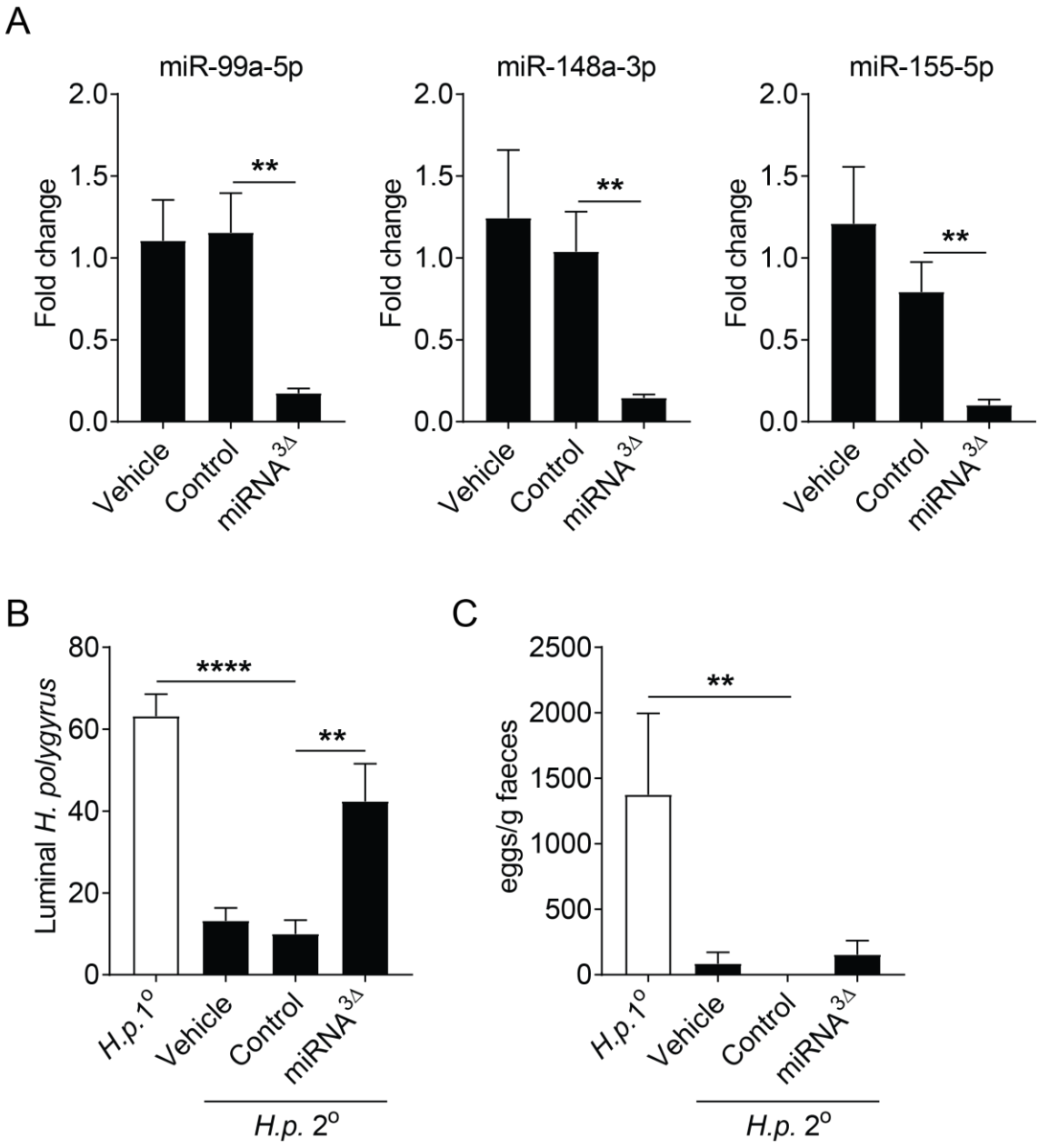


Figure 4.9 Concurrent triple miRNA inhibition abrogates immunity to 2º *H. polygyrus* infection

(**A**) miR-99a-5p, miR-148a-5p and miR-55-5p expression in the small intestine following vehicle, control inhibitor (control) or triple miRNA inhibitor (miRNA$^{3\Delta}$) treatment. (**B**) Luminal *H. polygyrus* adult worms in the small intestine and (**C**) faecal egg burden on day 49 (14 days-post 2º infection) following treatment vehicle, control inhibitor (control) or triple miRNA inhibitor (miRNA$^{3\Delta}$) treatment. Data represented as mean ± SEM, n=5. Data is representative of one experiment. ** = $p< 0.01$, **** = $p< 0.0001$ determined using a one-way ANOVA with Dunnett's multiple comparison analysis.

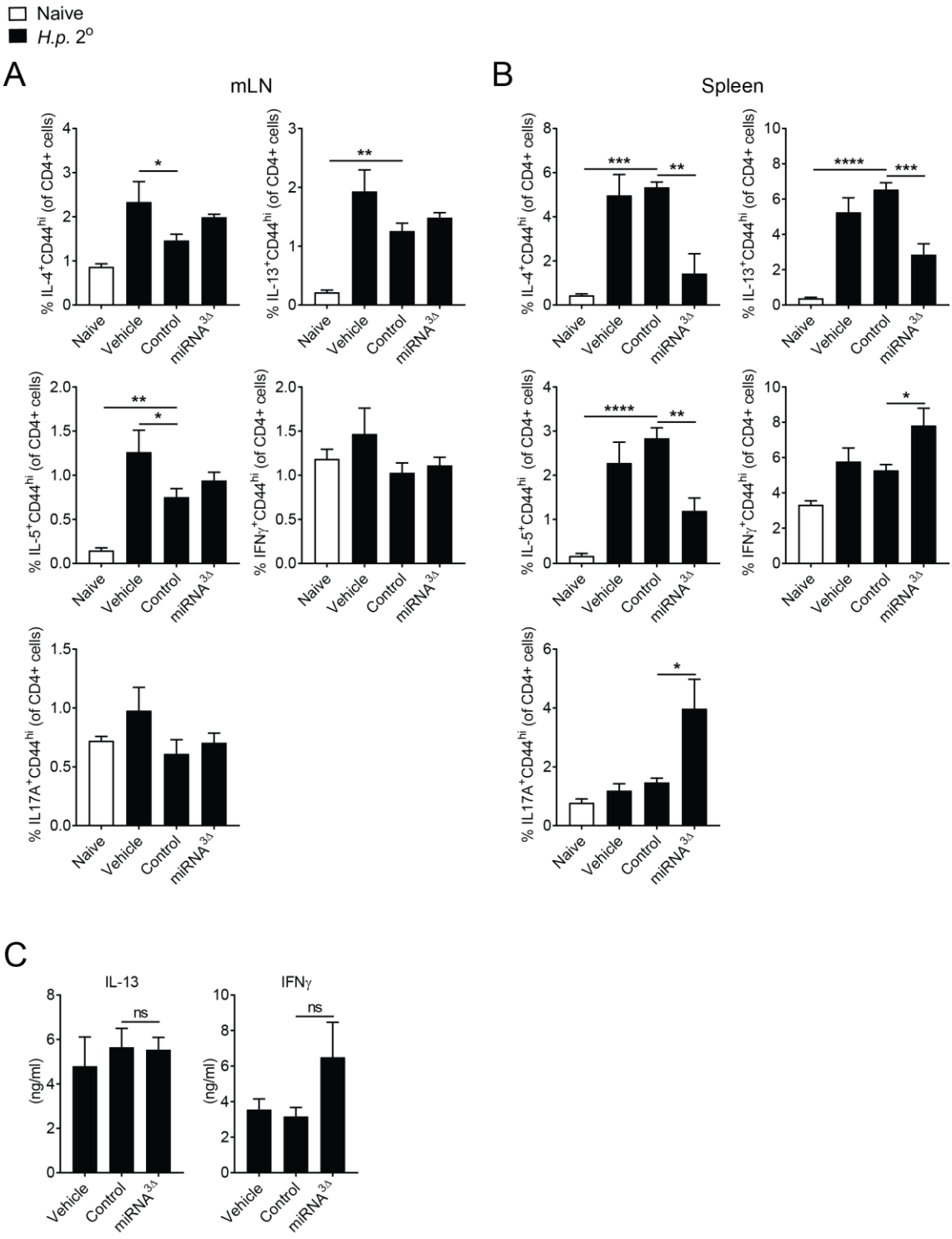

Naive
H.p. 2°
A
mLN
B
Spleen
% IL-4+CD44hi (of CD4+ cells)
% IL-13+CD44hi (of CD4+ cells)
% IL-5+CD44hi (of CD4+ cells)
% IFNγ+CD44hi (of CD4+ cells)
% IL17A+CD44hi (of CD4+ cells)
Naive
Vehicle
Control
miRNA3Δ
C
IL-13
IFNγ
(ng/ml)
ns

Figure 4.10 Concurrent triple miRNA inhibition alters the T cell response following 2º *H. polygyrus* infection

The frequency of cytokine producing $CD44^{hi}$ $CD4^{+}$ T cells in naive mice or 14 days-post 2º *H. polygyrus* infection following treatment with vehicle only, negative control inhibitor (control) or triple miRNA inhibitor ($miRNA^{3\Delta}$) in the mLN (**A**) and spleen (**B**). (**C**) *ex vivo H. polygyrus* antigen-specific cytokine production from the mLN 14 days-post 2º infection. Data represented as mean ± SEM, n=5. Data is from one experiment. * = $p<0.05$, ** = $p< 0.01$, **** = $p< 0.0001$ determined using a one-way ANOVA with Dunnett's multiple comparison analysis.

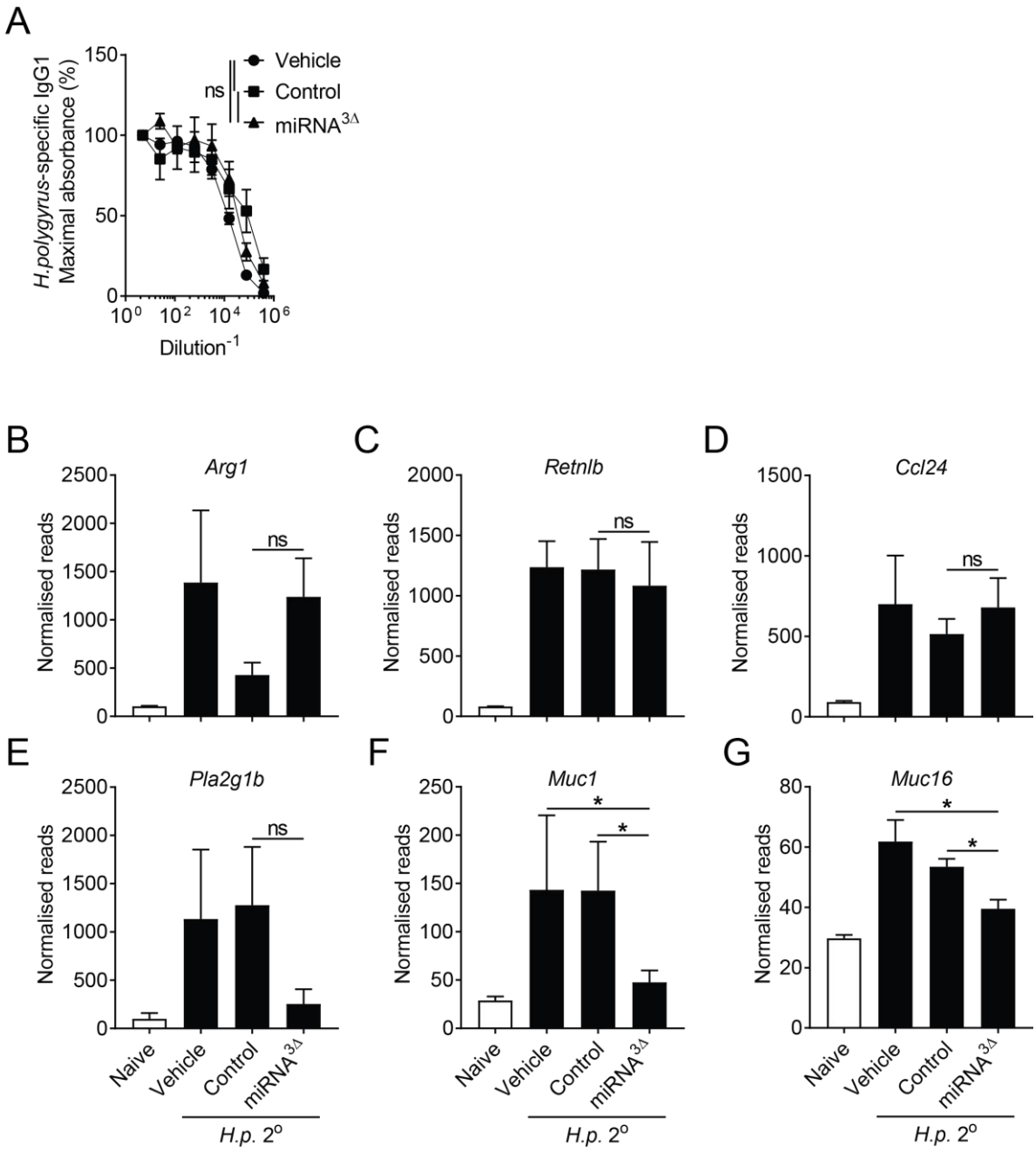


Figure 4.11 Concurrent triple miRNA inhibition inhibits mucin responses to *H. polygyrus*

(**A**) *H.* polygyrus-specific IgG1 in the serum from mice 14 days-post 2º *H.* polygyrus infection following treatment with vehicle only, negative control inhibitor (control) or triple miRNA inhibitors (miRNA$^{3\Delta}$). Expression of *Arg1* (**B**), *Retnlb* (**C**), *Ccl24* (**D**), *Pla2g1b* (**E**), *Muc1* (**F**) and *Muc16* (**G**) in the small intestine of naïve mice or 14 days-post 2º *H.* polygyrus infection following treatment with vehicle only, negative control inhibitor (control) or triple miRNA inhibitors (miRNA$^{3\Delta}$) determined by RNA sequencing. Data represented as mean ± SEM, n=5. Data is representative of one experiment. ns = not significant, * = p<0.05 determined using two-way ANOVA with Dunnett's multiple comparison analysis or by RNA sequencing analysis.

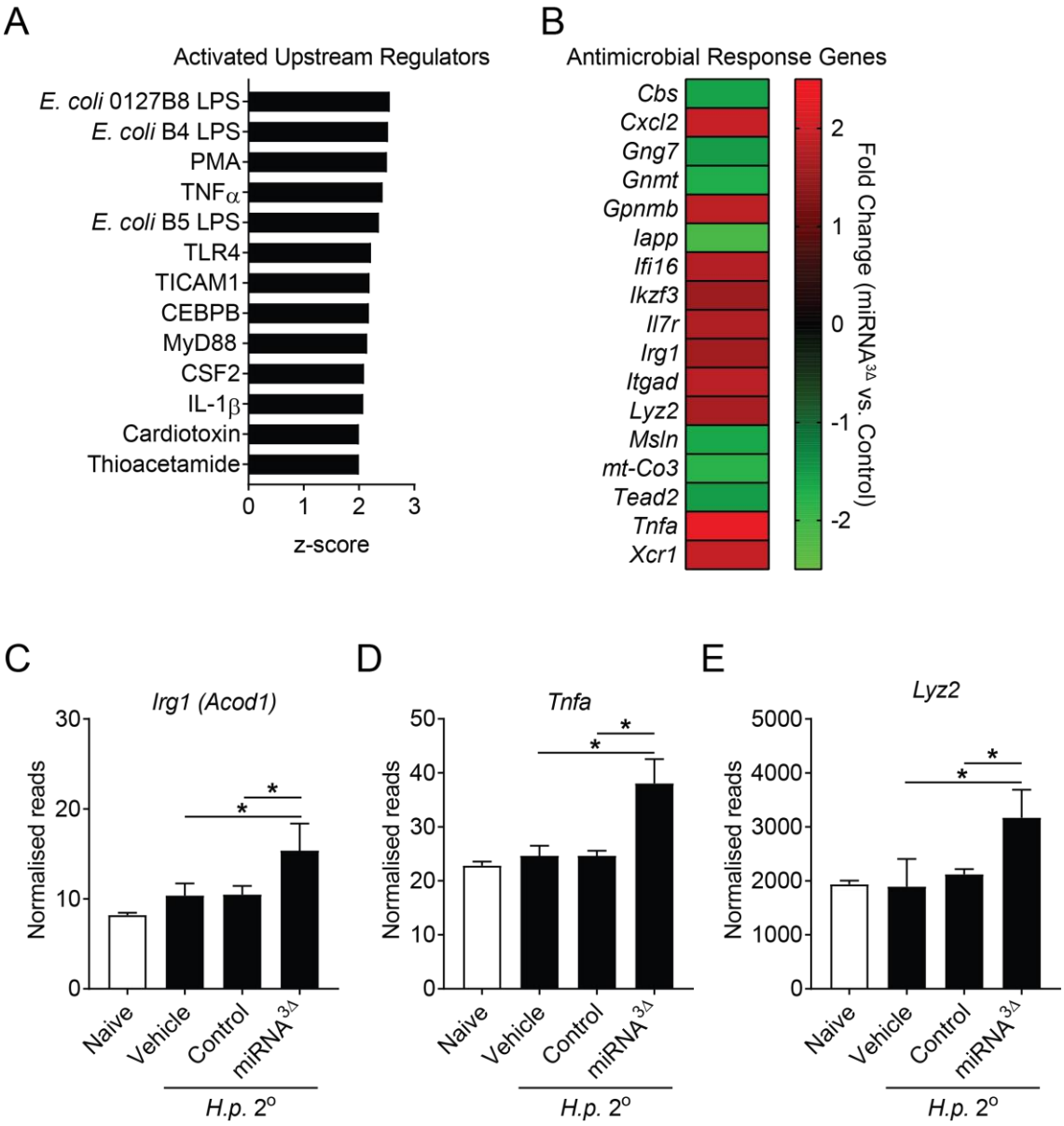


Figure 4.12 Transcriptome analysis identified an increased antimicrobial signalling signature following triple miRNA inhibition

(**A**) Predicted activated upstream regulators of the transcriptome of mice following triple miRNA inhibitor treatment (miRNA$^{3\Delta}$) (compared to control inhibitor treatment) 14 days-post 2^{o} H. polygyrus infection. (**B**) Fold change of the differentially expressed genes responsible for the predicated activation of the upstream regulators in (**A**) following miRNA$^{3\Delta}$ (compared to control inhibitor treatment, p<0.05, 1.5 fold change filter). Expression of the antimicrobial response genes *Irg1* (**C**), *Tnfa* (**D**) and *Lyz2* (**E**) in the small intestine of naïve mice or 14 days-post 2^{o} *H.* polygyrus infection following treatment with vehicle only, negative control inhibitor (control) or triple miRNA inhibitors (miRNA$^{3\Delta}$) determined by RNA sequencing. Data represented as mean ± SEM, n=5. Data is representative of one experiment. * = p<0.05 determined by RNA sequencing analysis.

Hypothesis: miRNAs miR-99a-5p, miR-148a-3p and miR-155-5p inhibit antimicrobial signalling which promotes protective immunity to *H. polygyrus*.

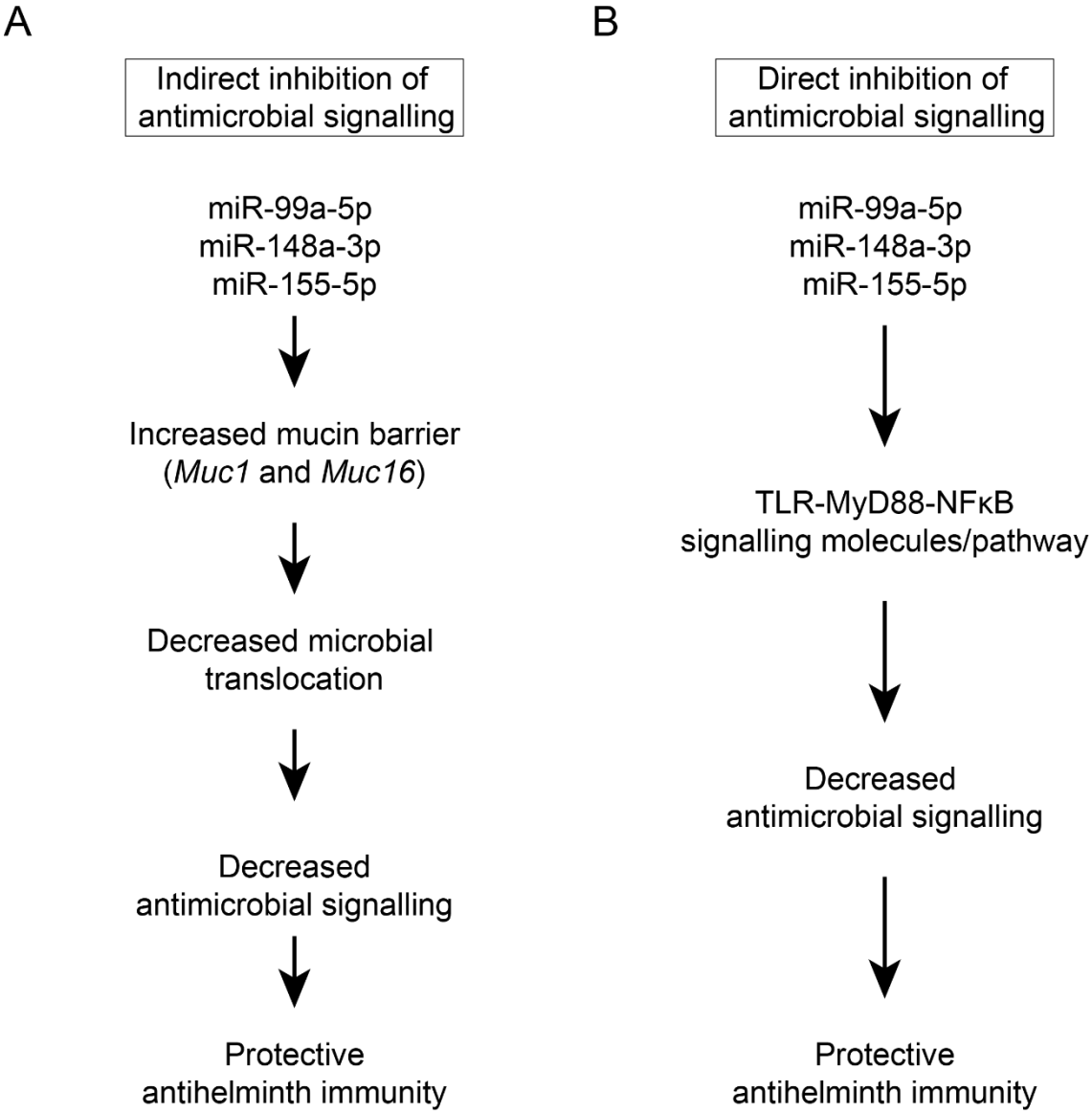


Figure 4.13 Hypotheses as to how miRNAs miR-99a-5p, miR148a-3p and miR-155-5p regulate antimicrobial signalling

(**A**) Indirect miRNA inhibition of antimicrobial signalling hypothesis. (**B**) Direct miRNA inhibition of antimicrobial signalling hypothesis.

Chapter 5. General discussion

Intestinal helminth infections are highly prevalent, infecting approximately a third of the world's population, and are responsible for significant health and economic burdens (Bartsch et al., 2016, Fitzpatrick, 2013, Bethony et al., 2006). With a small number of chemotherapeutic drugs available, evidence of drug-resistant helminths (Kaplan and Vidyashankar, 2012) and stalling vaccine efforts, it is imperative to improve our understanding of antihelminth immunity to identify new therapeutic avenues. Utilising next generation RNA sequencing, it is within this context that we have identified both two novel mechanisms of antihelminth immunity.

5.1 *H. polygyrus*: a suitable model for the study of antihelminth immunity?

The well-established *H. polygyrus* model of intestinal helminth infection was used in this thesis to identify novel mechanisms of antihelminth immunity. *H. polygyrus* is from the same phylogenetic order as the human hookworm parasites *N. americanus* and *A. duodenale,* as well as the ruminant parasites *H. contortus* and *T. circumcincta,* (Gouy de Bellocq et al., 2001) making *H. polygyrus* a suitable model of STH. Furthermore, *H. polygyrus* is a naturally occurring intestinal helminth of the mouse, establishing chronic infection in many inbred mouse strains (Ehrenford, 1954). In addition, *H. polygyrus* does allow for the study of intestinal antihelminth immunity in isolation, without the complexity of multiple organs. It is therefore a useful model for focussing on mechanisms of chronicity and resistance within the small intestine. Importantly, susceptible mice can acquire resistance to *H. polygyrus* following drug-cure of a 1^{o} infection, expelling subsequent 2^{o} challenge infections (Finkelman et al., 1997). This facet of the *H. polygyrus* model was essential in our investigation of novel antihelminth mechanisms, providing the comparison of susceptibility and resistance in the same genetic strain of mice.

However, there are caveats in utilising *H. polygyrus* for the study of antihelminth immunity. *H. polygyrus* is a strictly enteric parasite, like both ruminant helminths *H. contortus* and *T. circumcincta*, transmitted by the faecal-oral route (Ehrenford, 1954).

However, *N. americanus* and *A. duodenale* infect humans through skin penetration and migrate to the lung before entering the small intestine, much like the lifecycle of *N. brasiliensis*. Therefore, the strictly enteric *H. polygyrus* mouse model does not fully replicate the infective lifecycle of its human helminth relatives, suggesting that *N. brasiliensis* maybe a better model to study human STH. However, *N. brasiliensis* does not establish chronic infections in mice (Camberis et al., 2003), preventing its use to model chronic infection. Currently, there is not a naturally occurring mouse intestinal helminth which mimics the infective lifecycle of both *N. americanus* and *A. duodenale* and establishes chronic infection. A further caveat of the using *H. polygyrus* to model STH is the ability to acquire resistance. The ability of mice to acquire resistance to 2^{o} challenge infection following chemotherapeutic drug-cure of 1^{o} infection is rarely observed in human helminth infection. Although in the study of endemic populations identified that some individuals exhibit a greater degree of resistance that others, with 70% of the worm burden occurring in only 15% of the infected individuals (Hotez et al., 2008). Indeed, the generation of antihelminth immune memory takes years to develop and rarely achieves sterile immunity (McSorley and Maizels, 2012), unlike the classic immune memory-mediated protection observed against viral and bacterial pathogens (Farrell et al., 2017).

Nevertheless, investigating the mechanisms of acquired immunity to *H. polygyrus*, and other intestinal helminths, may uncover novel mechanism of immunity that could be translated into human antihelminth immunity, vaccine development and drug design. This is exemplified in this thesis, where we identified an endogenous anthelmintic, PLA_2g1B. Whether PLA_2g1B mediates the killing and expulsion of human and ruminant intestinal helminths remains to be tested.

It would be of great interest to employ a similar transcriptomic comparison strategy in other intestinal helminth models to identify compare and contrast mechanisms of antihelminth immunity. Utilising *T. muris*, which infects the cecum and colon, may uncover novel antihelminth mechanisms specific to whipworms and the large intestine. For example, *Pla2g1b*-deficiency compromised immunity to small intestine dwelling helminths, *N. brasiliensis* and *H. polygyrus*, but not the cecum dwelling *T. muris*. Specifically, the comparison of the cecum transcriptome of C57BL/6 mice following a low dose (susceptible) versus a high dose infection (resistant) may identify transcriptional changes associated with resistance. Furthermore, these

resistant-specific transcriptional changes could be cross-referenced to those identified here, both mRNA and miRNA, to determine both conserved and helminth species-specific mechanisms of antihelminth immunity.

5.2 Cooperation of type 2 immunity and the microbiota for functional antihelminth immunity?

Our understanding of immunity to intestinal helminths places type 2 immunity at forefront of protection, with type 1 immune responses contributing to susceptibility and chronic infection (Grencis, 2015, Allen and Maizels, 2011). The type 2 cytokines, IL-4 and IL-13, are critical in mediating many of the immunological and pathophysiological antihelminth effector immune responses (Urban et al., 1991b, Else and Grencis, 1996).

For protective immunity to *H. polygyrus*, $CD4^+$ T cell production of IL-4 and IL-13 is essential in mediating IL-4Rα-induced effector response (Urban et al., 1991a, Urban et al., 1991b). The IL-4Rα-dependent induction of *Arg1*-expressing aaMϕs (Anthony et al., 2006), Relmβ production from goblet cells (Herbert et al., 2009) and the production of antigen-specific IgG1 (McCoy et al., 2008) are critical for protection. In **Chapter 3** we demonstrated that the memory type 2 immune response is ineffective in mediating protection to 2^o *H. polygyrus* infection in the absence of PLA_2g1B. Furthermore, PLA_2g1B, when added to L3 larvae *in vitro*, was able to provide protection against 1^o *H. polygyrus* infection in the absence of a memory type 2 response. However, *in vivo* the induction and anthelmintic effect of PLA_2g1B required a functional immune system, suggesting a cooperation between the type 2 immune response and the direct anthelmintic properties of PLA_2g1B for functional protection. Interestingly, our data indicated that *Pla2g1b* expression in IECs was regulated by pathways distinct from type 2 immunity. Instead, *Pla2g1b* expression required intestinal microbiota, uncoupling a critical mediator of antihelminth immunity from type 2 immunity. The role of the microbiota in influencing immunity is not a new concept. Specific bacterial species and products have previously been demonstrated to shape distinct immune responses. For example, the generation of local Th17 cells in the intestine is dependent upon the presence of segmented filamentous bacterium

(Ivanov et al., 2009) and microbiota-derived butyrate is essential for the differentiation of colonic Tregs, thus regulating intestinal homeostasis (Furusawa et al., 2013). The exact mechanism in which *Pla2g1b* is regulated, beyond that described above, remains unclear. However, it is exciting to speculate that the current notion of antihelminth immunity maybe expanded beyond type 2 immune-mediated mechanisms, incorporating other immune and non-immune aspects, such as microbiota-mediated shaping of stromal immunity.

Contrary to the above postulation, increased microbial detection may in fact have a detrimental effect on functional antihelminth immunity. Indeed, previous studies demonstrated that *Myd88*-deficient mice, which have compromised antimicrobial recognition, were more resistant to a 1^{o} *H. polygyrus* infection (Reynolds et al., 2014a). Furthermore, we demonstrated that simultaneous inhibition of miR-99a-5p, miR-148a-3p and miR-155-5p resulted in increased activation of LPS-TLR4-MyD88 signalling cascades and abrogated resistance to 2^{o} *H. polygyrus* infection. These data can be extrapolated to suggest that resistant mice upregulate expression of miR-99a-5p, miR-148a-3p and miR-155-5p to inhibit antagonistic antimicrobial signalling to allow for unimpeded protective antihelminth immunity following 2^{o} challenge infection.

These two notions can be reconciled when considering the time and space of microbial interaction. The intestinal microbiota could be a positive influence on antihelminth immunity when the epithelial barrier function is not compromised. For example, following drug-cure of a 1^{o} *H. polygyrus* infection where the microbiota is required for the expression of *Pla2g1b*. However, upon infection, *H. polygyrus* larvae penetrate through the mucosal barrier of the small intestine, potentially allowing for the commensal microbes to translocate into the intestinal tissue, thus inducing the MyD88 signalling cascade and associated antimicrobial inflammatory responses. In this setting, the inflammatory response induced by the intestinal microbes may antagonise antihelminth immunity, preventing killing and expulsion (Reynolds et al., 2014a).

Although, type 2 immune responses are essential for antihelminth immunity, a coordinated response with the stromal compartment is essential. The influence of the microbiota with respect to antihelminth immunity is still poorly understood.

Nevertheless, as we show here, the microbiota was required for induction of the endogenous anthelmintic enzyme PLA_2g1B but it may also antagonise expulsion mechanisms. Identifying 'protective' components of the microbiota or their products that promote PLA2g1b may open up new areas of probiotic-related studies in the treatment of intestinal helminths

5.3 Tissue memory?

The design of our RNA sequencing experiments incorporated two control groups of drug-treated, resistant mice that were not given a 2^o challenge infection after drug clearance of the 1^o infection. These groups were denoted as Rx (D42) and Rx (D63), representing mice 28 days- and 49 days-post drug-mediated parasite clearance. Although not analysed in detail within this thesis, the mRNA and miRNA transcriptional profiles of groups Rx (D42) and Rx (D63) were markedly different from naïve mice (**Figure 3.1.C.** and **Figure 4.3.B**, respectively). Specifically, there was an increase in differentially expressed mRNA and miRNAs in these groups (Rx (D42) and Rx (D63)) compared to 1^o *H. polygyrus* infection (relative to naïve, p<0.05). These observations suggested that there was a new transcriptional 'baseline' in the small intestine in resistant mice, prior to 2^o challenge infection, which was maintained for up to 49 days-post drug-cure. Indeed, expression of *Pla2g1b*, miR-99a-5p and miR-148a-3p was upregulated in resistant mice, prior to 2^o challenge infection, and were required for protection against 2^o infection. Are these changes in the transcriptome of the small intestine therefore conferring tissue/stromal memory, distinct from classical adaptive immunological memory, providing protection against subsequent infection? If so, how is this tissue memory maintained? Is it through the alteration of the intestinal microbiota? Or is it mediated by the seeding of immune cells *in situ*, such as memory lymphocyte clusters (MLCs) (Iijima and Iwasaki, 2014, Schenkel et al., 2014). Furthermore, would this helminth-induced tissue memory alter the response to other pathogens?

A recent study has suggested that tissue or stromal memory exists in the skin, with inflammatory insults inducing epithelial stem cell memory for at least 180 days, hastening barrier restoration after subsequent tissue damage (Naik et al., 2017). In

this study, stromal memory was independent of macrophages or T cells, instead the 1º inflammatory insult induced intrinsic epigenetic changes in epithelial stem cells. In addition, mechanisms of local immunological memory have been demonstrated, involving the formation of macrophage-T cell MLCs (Iijima and Iwasaki, 2014, Ariotti et al., 2014, Schenkel et al., 2014). Whether MLCs influence or require tissue-derived factors, representing tissue memory, and whether local MLCs are induced following drug-cure of 1º *H. polygyrus* infection is unclear and warrants further study.

5.4 Lessons learnt and future applications

Throughout this thesis we have utilised RNA sequencing approaches in an attempt to identify novel mechanisms of antihelminth immunity. To this end, we identified PLA_2g1B as an endogenous anthelmintic, critical for immunity to both *H. polygyrus* and *N. brasiliensis*. We also identified a critical miRNA regulatory node, including miR-99a-5p, miR-148a-3p and miR-155-5p, which was critical for protective immunity to *H. polygyrus*.

For the defence against many classes of pathogens, the host has evolved endogenous molecules to directly impair and kill the invading pathogen, such as cathelicidins, defensins, histatins, lysozyme and lactoferrin (Zelechowska et al., 2016). We can now add the direct anthelmintic activity of PLA_2g1B to this arsenal of host-derived defence molecules. Further work should be focussed on elucidating the role of phospholipids in intestinal helminth health and infection, as well as investigating the precise mechanism by which *Pla2g1b* expression is regulated in the host intestine. Following these two lines of research could allow these finding to be translated into potential therapeutics; in the generation of a new class of synthetic anthelmintic drugs, which target critical phospholipids required for helminth health, or by inducing host *Pla2g1b* expression in the intestine, alongside vaccine efforts, to promote long lasting immunity. Care must be taken in manipulating host expression of *Pla2g1b* as mouse studies have demonstrated that PLA_2g1B can promote obesity, diabetes and atherosclerosis upon high calorie diet. Therefore, increasing *Pla2g1b* expression to aid antihelminth immunity may also promote diet-associated diseases. These postulations fall into an emerging field of research, the interplay between

metabolic syndromes and immunity. Human helminth infections have an inversely association with metabolic syndromes, such as type 2 diabetes and cardiovascular disease (Wiria et al., 2014), and animal models of STH suggest that helminth infection can improve insulin sensitivity following high fat diet (Wu et al., 2011). Further studies are needed to fully elucidate the relationship between intestinal helminth infection and metabolic syndromes. It would be interesting to study the interplay between the interplay between diet-induced obesity *H. polygyrus* infection, specifically investigating the role of PLA_2g1B in mediating both obesity and antihelminth immunity.

Over the past 16 years, since the discovery of miRNAs in mammals, the wave of excitement around miRNA-mediated gene regulation has peaked and slowly subsided. This may be, in part, due to the complex nature of miRNAs, far beyond the notion that a single miRNA regulates a single mRNA target, in regulating a suite of genes, much like transcription factors. As a result, miRNAs have been demonstrated to regulate several critical aspects of immunity, however their role in antihelminth immunity is relatively understudied. Here we identified three miRNAs, miR-99a-5p, miR-148a-3p and miR-155-5p, which act concurrently to promote resistance to *H. polygyrus*. Individual pharmacological inhibition of each miRNA did not abrogate immunity to 2^o challenge *H. polygyrus* infection, possibly highlighting a discrepancy between pharmacological transient inhibition and genetic constitutive knockout studies. Of significance, simultaneous inhibition of all three miRNAs did abrogate resistance. This may highlight an additional level of complexity of miRNA-mediated gene regulation, suggesting that miRNA species act in synergy to induce distinct biological functions. Further investigation into the localisation of these miRNAs in specific cell types would allow for comprehensive elucidation of the mRNA targets and mechanism of miRNA-mediated antihelminth immunity. These miRNAs are likely to be expressed in multiple cell types making this avenue of investigation particularly complex and difficult, requiring cell-specific targeted approaches to untangle the role of distinct miRNAs in specific cell types.

In conclusion, we have identified novel mechanisms of antihelminth immunity, the endogenous anthelmintic PLA_2g1B and a miRNA module critical in regulating the antihelminth immune response. These findings advance our understanding of

antihelminth immunity and help open up new areas of study in the ongoing quest for new therapeutics and avenues for the treatment of STH.

5.5 Figures

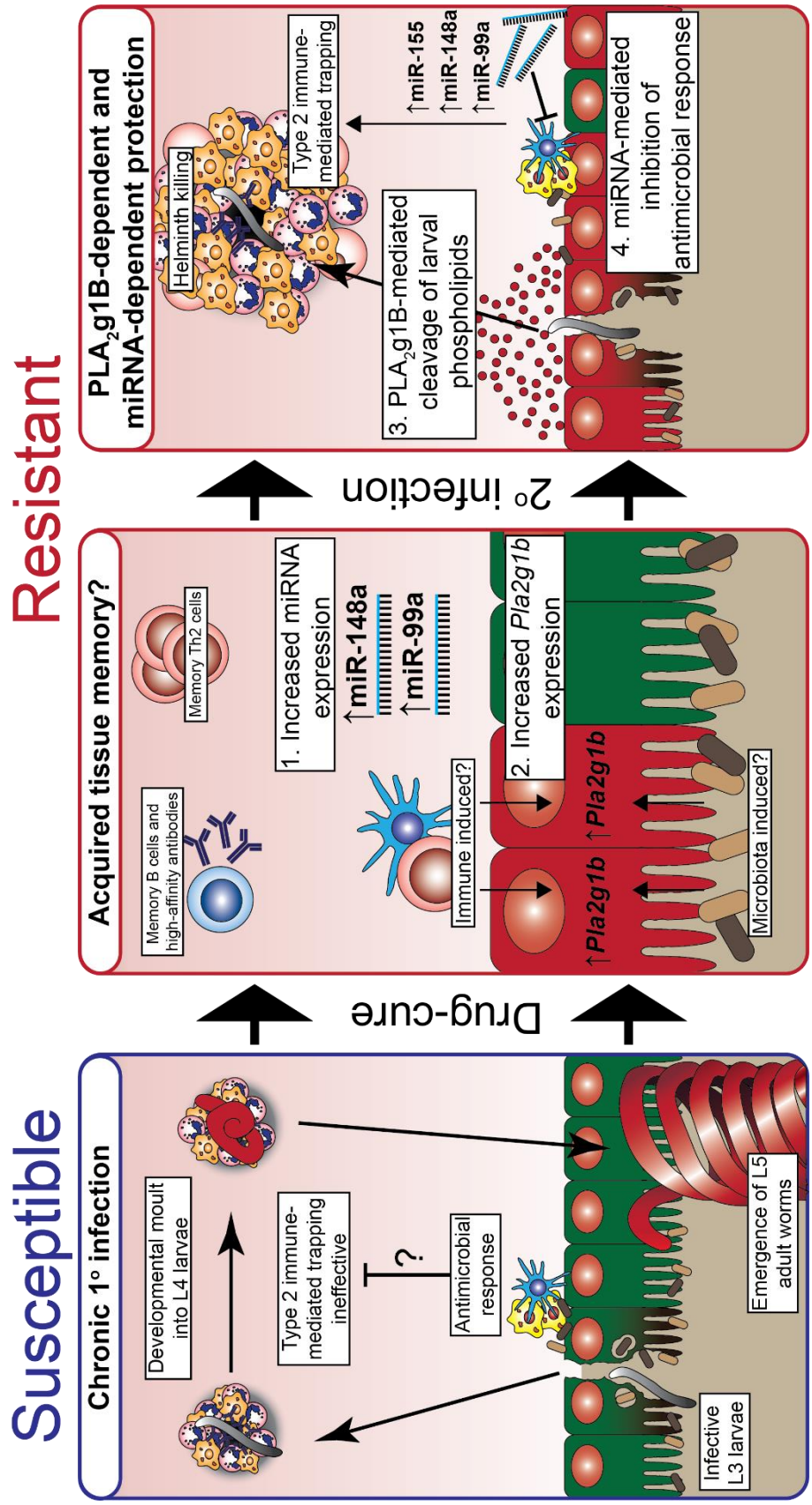

Susceptible
Resistant
Chronic 1° infection
Developmental moult into L4 larvae
Type 2 immune-mediated trapping ineffective
?
Antimicrobial response
Infective L3 larvae
Emergence of L5 adult worms
Drug-cure
Acquired tissue memory?
Memory B cells and high-affinity antibodies
Memory Th2 cells
1. Increased miRNA expression
↑miR-148a
↑miR-99a
Immune induced?
2. Increased Pla2g1b expression
↑Pla2g1b
↑Pla2g1b
↑Pla2g1b
Microbiota induced?
2° infection
PLA2g1B-dependent and miRNA-dependent protection
Helminth killing
Type 2 immune-mediated trapping
3. PLA2g1B-mediated cleavage of larval phospholipids
↑miR-155
↑miR-148a
↑miR-99a
4. miRNA-mediated inhibition of antimicrobial response

Figure 5.1 Working model: Expression of *Pla2g1b* and miRNAs miR-99a-5p, miR-148a-3p and miR-155-5p are upregulated in resistant mice and are essential for immunity to *H. polygyrus*.

C57BL/6 mice are susceptible to 1º *H. polygyrus* infection: Infective L3 larvae penetrate and embed into the small intestine where they undergo two developmental moults before emerging as adult L5 worms in the intestinal lumen.
Acquired tissue memory: Following drug-cure of 1º infection, a memory Th2 cells and memory B cells are established. In addition, the expression of miRNAs miR-99a-5p and miR-148a-3p are increased in the intestinal tissue (**Observation 1**). *Pla2g1b* expression is also upregulated in intestinal epithelial cells (**Observation 2**), mediated by both the immune compartment and/or the intestinal microbiota.
PLA_2g1B and miRNAs miR-99a-5p, miR-148a-3p and miR-155-5p are required for resistance to 2º challenge infection: PLA_2g1B directly cleaves phospholipids from invading L3 larvae (**Observation 3**). Anthelmintic PLA_2g1B acts in cooperation with type 2 immunity to promote helminth trapping and killing. Expression of miR-155-5p is increased in the intestinal tissue following 2º infection. Upregulation of miRNAs miR-99a-5p, miR-148a-3p and miR-155-5p is required to supress the antagonistic antimicrobial response (**Observation 4**) and promote functional type 2 immunity, resulting in helminth trapping and expulsion.

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Appendix

Publications

Entwistle LJ, Aegerter H, Czieso S, Chakravarty P, Nikolov N, Sesay A, Wilson MS. miR-99a, miR-148a and miR-155 establish a critical regulatory module maintaining antihelminth immunity. *Manuscript in preparation*.

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Awards

Scientific excellence in Molecular, Cellular and Immunoparasitology award winner, American Society of Tropical Medicine and Hygiene, 2016.

Best oral presentation, 20th Annual Woods Hole Immunoparasitology (WHIP) Meeting, Woods Hole, MA, USA. 18th – 21st April, 2016.

Best poster presentation, Molecular and Cellular Biology of Helminth Parasites IX, Hydra, Greece. 31st August – 5th September, 2015.

Oral Presentations

American Society of Tropical Medicine and Hygiene 65th Annual Meeting, Invited Speaker, Atlanta, Georgia, USA. 12th – 17th November, 2016. Title: *Epithelial cell-derived phospholipase A2 group 1B is an endogenous anthelmintic*.

20th Annual Woods Hole Immunoparasitology (WHIP) Meeting, Woods Hole, MA, USA. 18th – 21st April, 2016. Title: *Epithelial cell-derived PLA_2g1B is essential for immunity to Heligmosomoides polygyrus*.

Poster Presentations

18th Annual Congress of Mucosal Immunology, Washington D.C., USA. 19th – 22nd July, 2017. Title: *Epithelial cell-derived phospholipase A2 group 1B is an endogenous anthelmintic*.

20th Annual Woods Hole Immunoparasitology (WHIP) Meeting, Woods Hole, MA, USA. 18th – 21st April, 2016. Title: *Epithelial cell-derived PLA_2g1B is essential for immunity to Heligmosomoides polygyrus*.

Molecular and Cellular Biology of Helminth Parasites IX, Hydra, Greece. 31st August – 5th September, 2015. Title: *Endogenous phospholipase A_2 group 1B (PLA_2g1B) has direct anti-helminth properties and is essential for immunity to Heligmosomoides polygyrus*.